Antimicrobial resistance of *Escherichia coli* isolates from conventional and organic dairy farms; phenotypic and genotypic perspectives

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Omega Yaw Amoafo

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The use of antimicrobials has been the main weapon against infectious diseases for over a century. However, the persistent use/abuse of antimicrobials has resulted in resistant strains, and antimicrobial resistance (AMR) is a key health problem for health authorities and governments worldwide. This study aimed to shed light on the impact of antimicrobial usage and AMR development and prevalence by comparing the AMR status of *Escherichia coli* from the soils of conventional dairy farms to their organic counterparts in the Geraldine area of South Canterbury, New Zealand. Dairy farm soil *E. coli* (DfSEC) isolates (n=814) were phylogenetically typed and antimicrobial susceptibility tested (AST) against cefoxitin, cefpodoxime, chloramphenicol, ciprofloxacin, gentamicin, meropenem, nalidixic acid, and tetracycline. Twenty of the DfSEC isolates were selected based on their absolute resistance to cefoxitin 30 mg for bioinformatics analysis.

The 814 DfSEC isolates were classified into seven main *E.coli* phylogenetic groups. The phylogeny group B1 was most prevalent at 73.7%. The E group was next at 9.6% while the A was 5.8%, C was 5.3% and the D and B2 groups were 0.5% each. When classified under the newer *E. coli* phylogeny nomenclature, the cladeI+II and cladeIII+IV+V phylogroups were found to be less than 1%. There was no F phylogeny group member identified in this study and 3.1% of the isolates were placed as

unknowns. The AST results indicated 3.7% of the DfSEC isolates were resistant to at least one of the eight selected antimicrobials.

Of the selected 20 DfSEC isolates, each carried at least two resistant gene variants. Three isolates from a conventional farm carried up to six resistant gene variants. Each of the 20 isolates may encode nucleotide sequences of either the *bla*ACT-1, *bla*BIL-1, *bla*CMY-2, or the *bla*MIR-1 gene variants for extended-spectrum β-lactamase (ESBL) production and the multi-drug transporter gene *mdf*A1 for the efflux pump. Two isolates from different farms showed close relatedness (≤ 1 locus difference apart). Statistical analysis indicated that there was no significant difference ($P > 0.05$) between the effect of the two dairy farming systems on the AMR of the DfSEC isolates but isolates from the conventional dairy farm possessed more resistance gene variants per organism than those from the organic dairy farms. DfSEC isolates commonly associated with humans and their companion animals may be transferred into the dairy farm soil and there is the possibility of farm-to-farm transfer of DfSEC isolates. Virulent mastitis and/or metritis causing *E. coli* of phylogeny group A may be found in the dairy farm soil.

This study archived over 800 dairy farm soil *E. coli* isolates for future comparative studies and laid a foundation for a future study of the trend of antimicrobial resistance development and their spread in the central region of the South Island of New Zealand.

Keywords: *antimicrobial, cefoxitin, extended-spectrum, genomic, isolates, lactamase, multi-drug, phylogenetic, virulent, variant.*

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Dedication

This body of work is dedicated to my mentor, my insopirator, the person always willing to listen and help, always giving a shoulder to others. The calm, gentle and forever, smiling inspiring

Dr. Robyn Mcfarlane

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Symbols & Units	Meanings
%	Percentage
°	Degree
°C	Degrees Celsius
kg	Kilogram (10^3 gram)
g	Gram
mg	Milligram (10^{-6} kg)
µg	Microgram (10^{-9} kg)
L	Litre (10^3 mL)
mL	Millilitre
µL	Microlitre (10^{-9} L)
M	Moles/L
mM	Millimoles/L (10^{-3}Moles/L)
µM	Micromoles/L (10^{-6} Moles/L)
nM	Nanomoles/L (10^{-9} Moles/L)
km	Kilometer (10^3 m)
m	Meter
mm	Millimeter (10^{-3} m)
ha	Hectare (10^4 m^2)
h	Hour
min	Minute
s	Second
kbp	Kilobase pairs (10^3 base pairs)
bp	Base pairs
rpm	Revolutions per minute
A	Adenosine

Abbreviations	Meaning
Ab	Antibiotic
Ab	Antibiotic
AmpC	Class C beta-lactamase; Ambler class C
AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistance Gene
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BHI	Brain Heart Infusion
bla	Beta-lactam(*ase*)
bla*ACT*	Beta-lactamase AmpC type
bla*BIL*	Beta-lactamase named after patient, Bilial (first patient discovered from)
bla*CMY*	Beta-lactamase active on **c**ephamycins
bla*CTX*-M	Beta-lactamase active on cefotaxime, (first isolated in Munich)
blaDHA	Beta-lactamase discovered in Dhahran, Saudi Arabia
bla*SHV*	Beta-lactamase sulphydryl reagent variable
BLAST	Basic Local Alignment Search Tool
bla*TEM*	Beta-lactamase named after patient, Temoniera (first patient discovered from)
CDC	Center for Disease Control
CLSI	Clinical and Laboratory Standard Institute
CW	Clearwaters
C	Cytosine
DfSEC	Dairy farm Soil *Escherichia coli*
dH2O	Deionised water
DNA	Deoxyribonucleic acid
dNTP	Deoxyinucleoside triphosphate

Abbreviations	Meaning
ECDC	European Centre for Disease control
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended Spectrum Beta-lactam(*ase*)
ESR	Institute of Environmental Science and Research
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal Pathogenic *Escherichia coli*
FAO	Food and Agriculture Organisation
GDP	Gross Domestic Product
G	Guanine
KPC	Class A beta-lactamase; *Klebsiella pneumoniae* **c**arbapenemase
MBL	Class B beta lactamase; Metallo-beta-lactamase (oxidoreductase superfamily)
MIC	Minimum Inhibitory Concentration
MM	Molecular Marker
MRD	Mill Road
NDM	New Delhi Metallo-Beta-lactamase
OECD	Organisation for Economic Co-operation and Development
OXA	Class D beta-lactamase active on oxacillin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PF	Peel Forest
PNGM-1	Novel subclass B3 Metallo-beta-lactamase from a deep-sea sediment metagenome
RNA	Ribonucleic acid
T	Thymine

Abbreviations	Meaning
tRNA	Transfer ribonucleic acid
UTI	Urinary Tract Infection
WGS	Whole Genome Sequence
WHO	Word Health Organisation

Chapter 1

Introduction

Antimicrobial resistance (AMR) threatens to push modern civilization back into the pre-antimicrobial era of high morbidity and mortality rates of infections due to bacterial diseases (Brown & Nettleton, 2017; Venkat, 2019). Such a scenario would inevitably lead to harsh socio-economic difficulties caused by loss of trillions of dollars as a result of the loss of human and agricultural output and wastage of resources used to combat the effects of lack of therapeutic options in the face of bacterial infections (Jasovský, Littmann, Zorzet, & Cars, 2016; Naylor et al., 2018; Tacconelli & Pezzani, 2019). Friedman et al. (2016) summed up these negative impacts of AMR in terms of morbidity and mortality as; increased resource utilization and cost, alterations in guidelines in terms of loss of narrow-spectrum antimicrobial classes, and changes in established therapeutic regimes. The closures of wards in hospitals and cancellation of surgical procedures as chemo- and radiotherapy result in immune-compromised individuals are also some of the negative aspects of AMR (Teillant, Gandra, Barter, Morgan, & Laxminarayan, 2015; Willyard, 2017; Woolhouse, Waugh, Perry, & Nair, 2016). New Zealand (NZ) has not escaped bacterial resistance to antimicrobials which pose a threat to humans and animals (Heffernan, Bakker, & Williamson, 2014; Heffernan, Dyet, Munroe, et al., 2014; McMullan et al., 2016; Rademaker et al., 2020).

NZ is an agricultural country with dairy farming as one of the major contributors to its GDP and NZ's dairy industry contributes 3% of global dairy production with 21.2 billion litres of milk processed by dairy companies in 2018-2019 (DairyNZ, 2020). Conventional dairy farming, as opposed to organic dairy farming, contributes a significant amount of chemicals and their metabolites directly or indirectly to the environment (Chobtang, Ledgard, McLaren, & Donaghy, 2017; Mandal et al., 2020; Oliver et al., 2020; Singh et al., 2020). These two farming systems may therefore impact the microbiome in the soils differently (Groot & van't Hooft, 2016; Mandal et al., 2020; Zhang et al., 2020). The recent surge in AMR prevalence has been attributed to the exposure of soil microbes to

factors that cause evolutionary pressures for them to mutate and acquire resistance to survive, as they have done for millions of years (Allen et al., 2010; Finley et al., 2013; Gillings, 2013; Kim, Jensen, Aga, & Weber, 2007; von Wintersdorff et al., 2016).

It is prudent to monitor the population of microorganisms with broad resistance to antimicrobials in an environment, especially if they are closely associated with human and livestock activities (Argudín et al., 2017; Oliver, Murinda, & Jayarao, 2011; Wang, McEntire, Zhang, Li, & Doyle, 2012). In the case of *E. coli* found in dairy farm soils (DfSEC), it may be possible to evaluate the prevalence (Chee-Sanford, Aminov, Krapac, Garrigues-Jeanjean, & Mackie, 2001; Popowska et al., 2012) and the mode of transfer of antimicrobial-resistant strains or their genes horizontally or vertically (Geue et al., 2002; Lupo, Coyne, & Berendonk, 2012; Nesme & Simonet, 2015; Rivera et al., 2012). Bacteria strains carrying antimicrobial resistance genes (ARGs) may be transmitted from the livestock directly, by their products or through other environmental factors such as the soil, waterways, flooding, or insects as demonstrated in a study by Rybaříková, Dolejská, Materna, Literák, and Čížek (2010) to humans and vice versa (Collis et al., 2019; Hartmann et al., 2012; Heuer, Schmitt, & Smalla, 2011; Marti et al., 2013; van den Bogaard & Stobberingh, 2000)

1.1 Origins of antimicrobials

Raaijmakers and Mazzola (2012) citing Thomashow (2002) defined antimicrobials as heterogeneous, low molecular-weight compounds produced by microorganisms that inhibit the growth or metabolism of other microorganisms. This definition is similar to the definition of antimicrobials by Waksman (1947) who, by quoting Vuillemin (1889) on 'one creature destroying the life of another to sustain its own', defined antimicrobials as chemical substances produced by microorganisms that can kill or inhibit the growth of other microorganisms (Waksman, 1947). Lietman (1986) expanded this definition to include semisynthetic and synthetic antimicrobials with the term antibiotic, replaced by antimicrobial (Kourkouta et al., 2018).

It is widely accepted that ancient Chinese, Egyptians, Greeks, and Romans used extracts from fungi to cure various infections (Aminov, 2010; Gould, 2016; Kourkouta et al., 2018). The pioneering work of

Louis Pasteur and Robert Koch to link microorganisms to the cause of infectious diseases that had plagued humankind since the dawn of time (Gould, 2016), accelerated the search for a 'magic bullet' to cure infectious diseases (Aminov, 2010; Zaffiri, Gardner, & Toledo-Pereyra, 2012). Until the discovery of antiseptics in 1867, the reliance was mainly on the use of hygiene and sanitation for the prevention and spread of diseases (Jeśman, Młudzik, & Cybulska, 2011).

The discovery of mycophenolic acid secreted by *Penicillium brevicompactum* and used against *Bacillus anthracis* by the Italian physician and microbiologist, Bartolomeo Gosio in 1893 has been cited as the earliest antimicrobial discovered (Nicolaou & Rigol, 2018). Later in 1911, Paul Erlich discovered salvarsan for the cure of syphilis (Yazdankhah, Lassen, Midtvedt, & Solberg, 2013; Zaffiri et al., 2012). This was before the discovery of penicillin by Alexander Fleming in 1928 (Adedeji, 2016; Jeśman et al., 2011) with the period from 1950-1965 being described as the golden age of antimicrobial discovery (Gould, 2016; Kumazawa & Yagisawa, 2002).

The commercial production of antimicrobials in 1943 and the discovery of various other antimicrobials over time, to the present cephalosporins (Mohr, 2016; Patait et al., 2015; Zaffiri et al., 2012; Zaffiri, Gardner, & Toledo-Pereyra, 2013), have resulted in a huge improvement in dealing with infectious diseases (Figure 1.1).

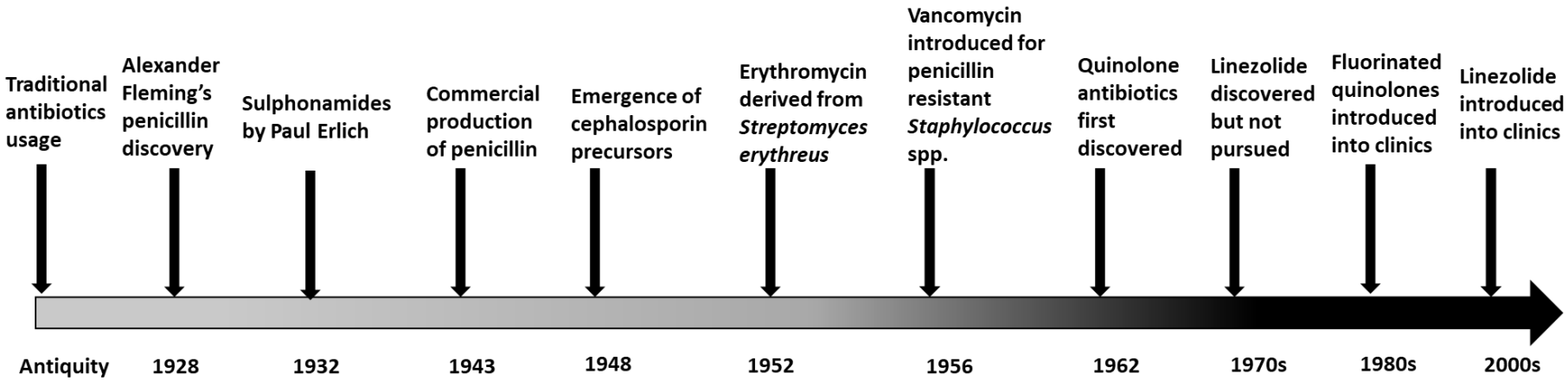

Figure 1.1 Timeline of antimicrobial discovery.

A modified version of chronology of antibiotic discovery and their introduction into clinics by (Patait et al., 2015).

1.2 The advent of antimicrobial resistance

According to The World Health Organisation (WHO), AMR develops when a microbe such as a bacterium, fungus, virus, or parasite can no longer be destroyed, or its growth is limited by a drug that the microbe was previously sensitive to. Such that, standard treatments no longer work. This leads to difficulties in treating or control of infections with prolonged hospital stays and increased risk of disease transmission, increased socioeconomic costs, and greater risks of deaths (World Health Organization, 2014). AMR, according to the WHO (2018) causes a 50% increase in risks compared to diseases caused by non-resistant microbes.

Bacterial AMR was predicted before the start of commercial antimicrobial production and usage because the genes that encode for AMR have been in existence long before their discovery and commercial production (D'Costa et al., 2011; Li et al., 2015; Martínez, 2012; Perry, Waglechner, & Wright, 2016; van Hoek et al., 2011; Wright & Poinar, 2012). In most cases, the discovery of resistance genes and their phenotypic expression occurred before or immediately following the mass production of a particular antimicrobial (Friedman et al., 2016). Aside from this, antimicrobial genes have also been found in regions and locations with little or no human interactions (Allen, Moe, Rodbumrer, Gaarder, & Handelsman, 2009; Lang et al., 2010; Ushida et al., 2010; Van Goethem et al., 2018). For example, Lee et al. (2019), citing earlier work carried out with Park et al. (2018), identified antimicrobial-resistant genes PNGM-1 in deep-sea sediments that are tens of thousands of years old. These two phenomena suggest that antimicrobials and resistance to antimicrobials have always been part of bacteria populations. Although, other studies have also indicated that the number of AMR genes found at these remote and pristine places suggests the role of wind and water currents and wildlife activities (Allen et al., 2010; Lang et al., 2010; Segawa et al., 2013; Ushida et al., 2010).

Antimicrobial resistance to a wide range of infectious agents is a huge public health threat (Aslam et al., 2018; Ferri, Ranucci, Romagnoli, & Giaccone, 2017; Frieri, Kumar, & Boutin, 2017; Laxminarayan, Duse, Wattal, Zaidi, Wertheim, Sumpradit, Vlieghe, Hara, Gould, Goossens, et al., 2013; World Health Organization, 2014). There has been a plateau in the discovery and emergence of effective

pharmaceuticals to combat microbial pathogens during the past couple of decades (Coates, Halls, & Hu, 2011; Hollis & Ahmed, 2014; Kalkreuter, Pan, Cepeda, & Shen, 2020; Lewis, 2013). For the past 50 years only one antimicrobial belonging to a new class, the narrow spectrum daptomycin, has been discovered and used in clinical settings (Fernandes & Martens, 2017; Lewis, 2012; Piddock, 2012).

Globally, the ever-increasing human population means agriculture has to keep up with food production (crop and animal) to sustain the various populations (Gilland, 2002; Laurance, Sayer, & Cassman, 2014). In most developed countries the call is for the elimination of highly intensive farming where animals are held indoors under controlled conditions (factory farming), as a source of food for human consumption (Foley et al., 2011; Garcia, Osburn, & Cullor, 2019; McMichael, Powles, Butler, & Uauy, 2007; Tilman, 1999). However most developing nations in Asia, Africa, and South America are urged to intensify their farming systems to produce enough food for local consumption or export (Carvalho, 2006; Duncan et al., 2013; Jayne, Chamberlin, & Headey, 2014). The use of antimicrobials is beneficial to animal factory farmers as there is an increase in productivity of the animals by the elimination of neonatal diseases and energy waste in fighting against pathogens (Angelakis, 2017; Gelbrand et al., 2015; Gorden & Plummer, 2010; Landers, Cohen, Wittum, & Larson, 2012). This, however, is the major way that humans have contributed to the present state of widespread resistance to clinical antimicrobials (Davies & Davies, 2010; Martin, Thottathil, & Newman, 2015; Mehdi et al., 2018; Sarmah, Meyer, & Boxall, 2006a; Watkins & Bonomo, 2016).

A Mexican study, however, found that the short-term deposition of sulphonamide and quinolone antimicrobials in the soil did not increase the relative number of sulphonamide (*sul*) and quinolone (*qnr*) resistance genes but long-term deposition increases the absolute concentration of these resistance genes in the soil (Dalkmann et al., 2012). A USA study of the metagenomics of AMR in the soils with a 14-year application of poultry and bovine manure containing antimicrobials and their metabolites found increased amounts of AMR genes in the soil compared to soils without antimicrobials (Yang et al., 2020). A Chinese study of the effect of antimicrobial and heavy metal usage in feed supplements and for disease control in the swine industry heavily correlated with the

abundance of antibiotic resistance genes (ARGs) in the soil of the swine farms (Zhu et al., 2013). Similarly, a Portuguese study into the effect of excreted antimicrobials, their derivatives, and metabolites on the occurrence and abundance of ARGs in wastewater indicated that the anthropological effect of antimicrobial release into the environment is to blame for the relative abundances of ARGs in the environment (Amador, Fernandes, Prudêncio, Barreto, & Duarte, 2015). A gradient study of a riverbed to ascertain the magnitude and effect of human activities on the occurrence and abundance of ARGs in the riverbed and suspended sediments indicated that the more antimicrobials, heavy metals, and other products of human activity that are deposited into the river at the various stations, the greater the occurrence of ARGs at the station (Pruden, Arabi, & Storteboom, 2012). A British Columbian study on the effect of the use of poultry litters heavily laden with antimicrobials and other agrochemicals as fertilizer for crop production farms correlated with the amount and occurrence of ARG in these soils (Furtula et al., 2010). Thus, the assessment that the development and prevalence of AMR and the spread of ARGs are due to the deposition of antimicrobials and their metabolites is well-founded.

Since bacteria have continually evolved to resist environmental stressors and pressures to preserve their existence for billions of years (Brockhurst, 2013; Durão, Balbontín, & Gordo, 2018; Esbelin, Santos, & Hébraud, 2018; Rodríguez-Rojas, Rodríguez-Beltrán, Couce, & Blázquez, 2013; van Heerwaarden, Kellermann, & Hoffmann, 2014), their adaptations to subvert antimicrobial therapy are not entirely surprising. The fronts of pressures that bacteria face are twofold; natural antimicrobials as indicated by the original definition of antibiotics. Secondly, the unquantifiable hundreds of millions of tons of antimicrobials and other pharmaceuticals that have been produced (Davies & Davies, 2010), eventually end up in the environment such as agricultural fields and water bodies as whole compounds or their metabolites (Angeles et al., 2020; Ghirardini, Grillini, & Verlicchi, 2020; Serra-Compte, Álvarez-Muñoz, Rodríguez-Mozaz, & Barceló, 2017; Yuan et al., 2019).

1.3 The extent of antimicrobial resistance

The global reduction in the discovery of new potent antimicrobials to deal with the rise and spread of AMR bacteria (Piddock, 2012; Roca et al., 2015) has been attributed to years of lack of global coordinated research and investment into the pharmaceuticals for new antimicrobials as well as actions to stop antimicrobial abuse (Hoffman et al., 2015; Inoue & Minghui, 2017; Laxminarayan, Sridhar, Blaser, Wang, & Woolhouse, 2016). Across the world, different organisations and governments have set up systems to deal with the threat that this situation poses (Chaudhary, 2016). In as much as the AMR patterns differ from country to country across the different regions of the world (Goossens, Ferech, Vander Stichele, Elseviers, & Group, 2005; O'Brien et al., 1978; Tacconelli et al., 2018; Tacconelli et al., 2019; Tandogdu et al., 2014), it is noted that the best way would be for a coordinated effort by all nations. This is because the problem is ever-increasing, and AMR does not restrict itself to political or geographical borders (Carlet et al., 2012; Chaudhary, 2016; Klein et al., 2018; Laxminarayan, Duse, Wattal, Zaidi, Wertheim, Sumpradit, Vlieghe, Hara, Gould, & Goossens, 2013; Tacconelli et al., 2018; Ventola, 2015).

The WHO global action plan on AMR (Hoffman et al., 2015; World Health Organization, 2018) is a step in a direction to find some coordination amongst member states. Secondly, the USA federal government's Center for Disease Control (CDC) One Health Office, is one of the major organisations which has been set up across different regions, sectors, and disciplines to combat the global AMR problem (Cipolla, Bonizzi, & Zecconi, 2015; Frieden, 2010). In Europe, the European Centre for Disease Prevention and Control (ECDC) (Mazińska, Strużycka, & Hryniewicz, 2017; Weist & Högberg, 2016), in North America, USA CDC and the Canadian government (Parmley et al., 2013; Zhanel et al., 2010), in Africa, the South Africa government (Osei Sekyere, 2016; Rweyemamu et al., 2013) have set up bodies that survey and regulate the use of antimicrobials and facilitate communication between the various antimicrobial stakeholders. These bodies collect data, set up surveillance networks, share information, educate, and liaise with lawmakers, medical, veterinary, and agricultural bodies to help

control the threat that AMR poses globally (Asokan & Vanitha, 2018; Mitchell et al., 2020; Purohit et al., 2017; Robinson et al., 2016; Rousham, Unicomb, & Islam, 2018; Wegener).

In NZ, the control of antimicrobial usage in agriculture and veterinary is by the Ministry for Primary Industries with advice from the NZ Veterinary Council, Agricultural Compounds and Veterinary Medicines group, and other crown and academic research institutions (Anon, 2020). To promote NZ's contribution to global antimicrobial stewardship, NZ is a member of the World Health Assembly's Tripartite Action Plan to tackle AMR formed by the WHO and the Food and Agriculture Organization (FAO) (Iossa & White, 2018). This assembly aims to ensure the treatment of infectious diseases successfully for as long as possible with effective and safe medicines that are quality assured, responsibly used, and easily accessible in times of need (Ministry of Health and Ministry for Primary Industries, 2017). This is an important undertaking for NZ because according to Harrison et al. (2020), despite the island nation's status of NZ which gives its ecosystem some isolation, the relatively high reliance of the nation's economy on agriculture causes a high incidence of infectious diseases. Notwithstanding the tight controls to prevent indiscriminate use of antimicrobials (Ministry of Agriculture and Fisheries, 2011), microbial resistance to antimicrobials continues to occur and present a danger to animals and humans in NZ (Heffernan, Bakker, et al., 2014; Heffernan, Woodhouse, Pope, & Blackmore, 2009; Loh, Vukcevic, & Bastos Gomes, 2020). For example, a report released by The NZ Institute of Environmental Science and Research (ESR) indicated that extended-spectrum β-lactamase *E. coli* (ESBL) isolated from human blood cultures increased from 2.6% during the period 2006-2008 to 3.8% in the period 2009-2011, an increase of 46% (ESR, 2012) and the prevalence rate of methicillin-resistant *Staphylococcus aureus* (MRSA) has increased by 78% from 13.4 to 23.9 per 100 000 persons over 10 years (2004-2013) (ESR, 2014).

1.4 Antimicrobial resistance mechanisms and their transfer

1.4.1 Penicillins

The contribution of penicillin to health and well-being, especially during and after WWII was immense and the tag of a wonder drug was not an overstatement at the time. However, even in 1940, before the mass production of penicillin, Abraham and Chain (1940) confirmed the observation made by Alexander Fleming in 1929 that certain bacteria can grow in the presence of penicillin. Penicillin belongs to the group of antimicrobials with a β-lactam ring at the centre of their structure. The β-lactam binds to a serine site of the penicillin-binding protein (PBP) (Brem et al., 2016; Yeats, Finn, & Bateman, 2002; Zervosen, Sauvage, Frère, Charlier, & Luxen, 2012) of bacteria belonging to the Ambler classes A, C, and D (Etemadi, Ebrahimzadeh Leylabadlo, & Ghotaslou, 2020). The ease of binding is because of the close resemblance to the D-alanyl-D-alanine substrate of transpeptidases involved in peptidoglycan synthesis. The ability of β-lactam to bind to the PBPs, disrupts the synthesis of peptidoglycan (Nordmann, Dortet, & Poirel, 2012a; Strominger, Blumberg, Suginaka, Umbreit, & Wickus, 1971; Sullivan, Delgado, Maharjan, & Cain, 2020; Tomasz, 1979), the key component of a bacteria cell wall, leading to cell lysis (Cavallari, Lamers, Scheurwater, Matos, & Burrows, 2013; Cho, Uehara, & Bernhardt, 2014; Tomasz, 1979; Zeng & Lin, 2013).

Resistance to β-lactam antimicrobials is mainly by the breakdown of the lactam ring by an enzyme β-lactamase to the biologically harmless penicilloic acids (Chakraborty, 2016; Fisher, Meroueh, & Mobashery, 2005; Ohalete, 2016; Peterson, Petrasky, Seymour, Burkhart, & Schuiling, 2012). Other mechanisms include a) pumping the drug out of its cell (Das, Verma, Kumar, Ghosh, & Ramamurthy, 2020; Levy, 2002; Mahamoud, Chevalier, Alibert-Franco, Kern, & Pagès, 2007), b) change in the structure of the target site of the drug (Lingzhi et al., 2018) or c) reduced permeability of the bacteria cell to the drug (Bryan, 1988; Masi, Réfregiers, Pos, & Pagès, 2017; Neu, 1982; Neu, 1992; Pagès, James, & Winterhalter, 2008).

Most Gram-negative bacteria, including opportunistic pathogens like *Escherichia coli* and other Enterobacteriaceae, have mobile genetic elements to produce β-lactamase for the breakdown of β-

lactam antimicrobial to ineffectiveness (Tooke et al., 2019). The ability of the β-lactam antimicrobial to disrupt the cell wall synthesis of a bacteria may also be reduced if the targeted site is modified in the bacteria (Singh, Sripada, & Singh, 2014; Todar, 2011; Yao & Rock, 2016). As suggested by Fontana, Ligozzi, and Satta (1996) and Duezt et al. (2001), mutation at sites on resistant bacteria's genetic elements causes the over-production of low-affinity penicillin-binding proteins (PBP2x, PBP2b, or PBP1a). These low-affinity membrane-bound serine transferases of PBPs prevent the antimicrobial from disrupting the peptidoglycan formation so the bacterial cell wall's integrity is not compromised (Engel et al., 2014; Łeski & Tomasz, 2005).

4.1.1 Forms of penicillin

The realisation that penicillin in its natural and original structure has limitations, led to more studies and efforts into the chemistry and biochemistry of the compound which resulted in the discovery of different forms and groups of the antimicrobial (Clark, 2011; Clarke, 2015; Kardos & Demain, 2011). Different forms of penicillin such as benzylpenicillin, penicillamine, penaldic acid, 2-pentenylpenicillin, and other chemical formulations that varied in stability, solubility, and their acid-base properties (Clarke, 2015) were discovered/or developed. Other groups of antimicrobials that have this β-lactam ring in their structure include carbapenems, monobactams, and cephalosporins (Lingzhi et al., 2018).

Carbapenem antimicrobials are forms of bicyclic β-lactam antimicrobials that differ from the penicillins in having a carbon atom replacing sulphur in the β-lactam ring (Berks, 1996; Karaiskos & Giamarellou, 2020; Mori, Takahashi, & Mizutani, 2007; Pham et al., 2020). The various carbapenems vary in their solubility, stability in solution or solid form, level of toxicity (Liang, Chen, & Macy, 2019) to mammals, and also in spectra of effectiveness (Moellering Jr, Eliopoulos, & Sentochnik, 1989; Park et al., 2014; Singh, Young, & Silver, 2017). Due to the variation in their toxicity such as allergic seizures and nephrotoxicity and stability, not all carbapenems discovered are clinically available (Salmon-Rousseau et al., 2020). Clinically available ones include imipenem and meropenem (Fernandes, Amador, & Prudêncio, 2013; Moellering Jr et al., 1989; Singh et al., 2017). Although carbapenems are stable against β-lactamases, and, even broad-spectrum β-lactamases (ESBLs),

resistance was discovered in 1993 and spread fast due to easy acquisition and transfer of carbapenemase genes through plasmids (Carattoli, 2011; Kim et al., 2020). The genetically and biochemically diverse inducible carbapenem hydrolysing β-lactamases are responsible for the resistance observed in this group of antimicrobials (Hu, Zhang, & Gu, 2020; Queenan & Bush, 2007; Rasmussen & Bush, 1997; Streling et al., 2018). This group of enzymes may even hydrolyse other β-lactams such as cephalosporins and penicillins even more efficiently than they do carbapenems (Amjad et al., 2011; Baxter & Lambert, 1994; Lee, Lu, et al., 2019; Mehta, Rice, & Palzkill, 2015; Rasmussen & Bush, 1997).

Monobactams are monocyclic β-lactam antimicrobials whose chemical structure mimics the iron-chelating siderophores that bacteria use to chelate iron from their host into their cells (Li, Oliver, & Townsend, 2017; Sykes, Bonner, Bush, Georgopapadakou, & Wells, 1981). The monobactams thus have easier penetration by the Trojan-horse mechanism into the bacteria cell to bind to the PBPs in the periplasm of the bacteria cell and disrupt protein synthesis (Kou et al., 2018; Mitton-Fry et al., 2012; Scorciapino et al., 2017). They are also the only group of β-lactam antimicrobial-resistant against metallo-β-lactamases antimicrobial inhibitors (Page et al., 2011). The wide use of the less toxic monobactam, aztreonam was followed by the development of resistance, especially the CTX-M-15 type cefotaxime hydrolysing β-lactamases (Fernandes et al., 2013; Maryam & Khan, 2018).

The cephalosporins are bactericidal β-lactam antimicrobials of different generations, first isolated from the fungus *Acremonium* (*Cephalosporium*) (Braga & Lackner, 2017; Shahbaz, 2017). Most cephalosporins in use presently are synthetic or semisynthetic. The effectiveness of first-generation cephalosporins is towards aerobic Gram-positive organisms (Curtis, Boley, Walls, Hamory, & Schmaltz, 1993; Zaffiri et al., 2012). Higher generations cephalosporins had improved and wider spectra of activity (Alexopoulou et al., 2013) and even used for the treatment of typhoid fever, a Gram-negative bacterial disease (Klemm et al., 2018; Soe & Overturf, 1987). Cephalosporins are well tolerated by human cells and show little toxicity (Vigliotta et al., 2020). The fifth-generation cephalosporins such as aztreonam, ceftaroline (Corey et al., 2010; File Jr et al., 2010; Lin et al., 2013), ceftobiprole (Nisha, Vanishree, & Mahesh, 2017; Selvan & Ganapathy, 2016), and cefoxitin

(Fernandes et al., 2013) on their own are effective against MRSA with relatively fewer side effects compared to other drugs and drug cocktails (Lin et al., 2013; Nisha et al., 2017; Selvan & Ganapathy, 2016).

1.4.2 Aminoglycosides

The aminoglycosides of which streptomycin (1944), kanamycin (1958), gentamicin (1963), and amikacin (semi-synthetic, 1972) are commonly known (Davies, 2007). They act by restricting protein synthesis for the growth of sensitive bacteria (Hancock, 1981; Wilton, Charron-Mazenod, Moore, & Lewenza, 2016). The antimicrobial binds to the 30S ribosomal subunit (Ribeiro da Cunha, Fonseca, & Calado, 2019) of the bacteria and stops the process of translation during protein synthesis (Jana & Deb, 2006). The wider use and importance of the aminoglycosides is explained by their broad-spectrum in being active against aerobic Gram-negative bacilli and Gram-positive cocci, ability to kill bacteria quickly and synergise with other drugs (Davies, 2007; Gunics, Motohashi, Amaral, Farkas, & Molnár, 2000; Hu et al., 2015; Jana & Deb, 2006; Krause, Serio, Kane, & Connolly, 2016).

Bacteria become resistant to gentamicin and other aminoglycosides by phosphorylation (Muir & Wallace, 1979), acetylation (Ishigaki et al., 2017), methylation (Reis, Kolvenbach, Nunes, & Corvini, 2020; Sun et al., 2016), changing antimicrobial cleavage site by nucleotide mutation of the16S rRNA (Pantel et al., 2018; Wong, Hendrix, Scott Priestley, & Greenberg, 1998), or efflux pump (Calvopiña, Dulyayangkul, & Avison, 2020; Hocquet et al., 2003). Bacteria may also acquire resistance by gene transfer as a transposon (Davies & Davies, 2010; Fenton, Harsch, & Klein, 1973; Honoré & Cole, 1994; Reis et al., 2020). The existence of both plasmid (Acar, Bulut, Stasiewicz, & Soyer, 2019; Guerra, Soto, Helmuth, & Mendoza, 2002; Oliva et al., 2018) and chromosomal genes (Miriagou, Carattoli, & Fanning, 2006; Sun et al., 2018) encoding for the resistance to aminoglycosides makes for easy transfer of these genetic materials between bacteria by both horizontal and vertical means. Some of the genes that encode for aminoglycoside modification enzymes (AMEs) secreted by resistant bacteria include *aac(3)-Ia, aac(6')-Ib, ant(4')-IIa, ant(2")-Ia, and aph(3")-Ib* (Ojdana et al., 2018).

According to Salih & Gibree (2019), gentamicin is the best antimicrobial for the treatment of cow mastitis caused by bacteria, and Sato, Bartlett, Kaneene, & Downes (2004) showed that out of 332 strains of *Campylobacter* spp., all were susceptible to gentamicin but not to ciprofloxacin or tetracycline. An Argentinian study found only 0-2.1% of *S. aureus* strains from cows with clinical and non-clinical mastitis to be resistant to a group of aminoglycosides but 48.4 % of them were resistant to penicillin-based antimicrobials (Russi, Bantar, & Calvinho, 2008). In a Turkish study on bacteria found in dairy products, 0%, 42%, and 55% of *Bacillus cereus*, *S. aureus,* and *E. coli* isolates were resistant to gentamicin, compared to 91%, 93%, and 90% to ampicillin and 0%, 54% and 66% to tetracycline, respectively (Gundogan & Avci, 2014). While in a surveillance study of 132 *E. coli* isolates from 110 dairy cows from 10 farms in New York State, 98.45% were resistant to ampicillin, 34.1% to streptomycin, 24.8% to tetracycline, and < 20% to gentamicin (Srinivasan et al., 2007).

In the NZ conventional dairy industry, aminoglycosides are rarely used (Ministry for Primary Industries, 2019). Aminoglycosides together with tetracyclines, macrolides/lincosamides or clavulanic acid made up less than 1% of the total intramammary veterinary antimicrobial sales in 2017 compared to 83% for penicillin and 14% of cephalosporin–based antimicrobials (Ministry for Primary Industries, 2019). The sales of aminoglycosides had reduced by 17% from 1 870 kg in 2016 to 1 557 kg in 2017. Data over five years indicated that sales of aminoglycosides increased to 2 611 kg in 2015, but declined annually following that period (Ministry for Primary Industries, 2019).

1.4.3 Quinolones

Nalidixic acid, the first of the quinolone group of antimicrobials, was serendipitously discovered in 1962 during the production of the antimalarial drug, chloroquine (Andersson & MacGowan, 2003; Bisacchi, 2015; Kuhlmann, Dalhoff, & Zeiler, 2012). The comparative ease of manipulation of the chemical structure of the quinolones has led to the discovery of many more antimicrobials of the group, which includes ciprofloxacin (Andersson & MacGowan, 2003; Appelbaum & Hunter, 2000). These changes in the chemical structure led to improvements in their potency against Gram-positive and Gram-negative bacteria, most notably Enterobacteriaceae such as *E. coli* and *Klebsiella* spp., and

atypical bacteria such as *Mycoplasma pneumoniae*. These changes also improved the tolerance of the drug by macro-organisms (Andersson & MacGowan, 2003; Appelbaum & Hunter, 2000). Quinolones were licensed for therapeutic use in1967 as drugs for the treatment of urinary tract infection caused by Gram-negative bacteria apart from *Pseudomonas aeruginosa* (Emmerson & Jones, 2003; Kuhlmann et al., 2012).

Quinolones are synthetic, meaning naturally occurring resistant genes did not exist in soils and the environment as is the case of other groups of antimicrobials (Hernández, Sanchez, & Martínez, 2011). However, a multidrug resistance efflux pump, inherent in most bacteria species (Blair & Piddock, 2016; Piddock, 2006) accounts for intrinsic resistance displayed by bacteria species and for the initial resistance to nalidixic acid demonstrated by Gram-positive bacteria such as *S. aureus* and *Streptococcus* spp. (Hernández et al., 2011).

Additional resistance mechanisms to the quinolones include target protecting proteins, and quinolone modifying enzymes. Genes encoding these proteins and enzymes are found on plasmids making them horizontally transferable (Hernández et al., 2011). The plasmid encoding the *qnr* gene protects the bacteria from DNA gyrase (Hernández et al., 2011) and reduces drug accumulation due to the efflux pump (Martínez-Martínez, Pascual, & Jacoby, 1998). The DNA gyrase prevents the supercoiling replication, transcription, recombination, and repair of DNA (Emmerson & Jones, 2003; Loganathan et al., 2017), which leads to cell death (Hernández et al., 2011).

The quinolones have been widely used in human medicine for the treatment of urinary tract infections caused by enteric bacteria (Aldred, Kerns, & Osheroff, 2014; Emmerson & Jones, 2003; Smithson et al., 2012; Stuck et al., 2012) and other therapeutic manipulations (Berning, Krasz, & Miehlke, 2011; Harris, Elhassan, & Flook, 2016), in veterinary medicine (Teuber, 2001; Wegner & Engberg, 2003) and other agricultural industries (Hou et al., 2015; Li et al., 2012; Quesada, Paschoal, & Reyes, 2013). These human activities have led to various amounts of the quinolones being found in various environmental niches (Oliveira, Nunes, Barreto Crespo, & Silva, 2020; Watkinson, Murby, Kolpin, & Costanzo, 2009). Up to 22 µg/kg of nalidixic acid was found in agricultural soils in a French

surveillance study (Tamtam et al., 2011). The incidence in resistance to nalidixic acid in veterinary sourced *Salmonella* strains in Germany increased from 0.2% in 1986 to 14.4% in 1996 (Malorny, Schroeter, & Helmuth, 1999). A similar study looking at the incidence in *Campylobacter* strains in the Netherlands found an increase in prevalence from 0% to 14% in seven a year from 1982 to 1989 (Endtz et al., 1991).

1.4.4 Tetracyclines

The tetracyclines were discovered, patented, and commercialised from the late 1940s to the early 1950s (Nelson & Levy, 2011b). Since then, modifications and derivatives have been developed/identified to overcome the bacterial resistance that was observed to earlier tetracyclines (Liu & Myers, 2016; Nelson & Levy, 2011a). Tetracyclines are widely used in humans (Daghrir & Drogui, 2013; Gu, Walker, Ryan, Payne, & Golub, 2012; Swamy, Sanivarapu, Moogla, & Kapalavai, 2015) and veterinary (Chantziaras, Boyen, Callens, & Dewulf, 2014; Eliopoulos, Eliopoulos, & Roberts, 2003; Jones, Woolfson, Djokic, & Coulter, 1996; Margolis, Fanelli, Hoffstad, & Lewis, 2010; Prescott, 2017) medicine, in agriculture for the treatment of diseases of plants, such as *Mycoplasma* spp. infection in palm trees (Maramorosch, 2012; McCoy, 2012), *Xanthomonas campestris* of seeds (Yemata & Fetene, 2017) and black rot in crucifers (Schaad & Dianese, 1981; Singh, Rathaur, & Vicente, 2016) and in aquaculture (Pereira, Silva, Meisel, & Pena, 2015; Tuševljak et al., 2013). Tetracyclines are classified as broad-spectrum, bacteriostatic antimicrobials of both natural and semisynthetic origins (Nelson & Levy, 2011b; Tariq, Rizvi, & Anwar, 2018). Tetracyclines, in human medication, have been widely used in periodontal disease management and treatment (Bokor-Bratić & Brkanić, 2000; Jones et al., 1996; Masoumi, Andisheh-tadbir, Firozabadi, Bahmanpour, & Tanideh, 2017; Rosenstein, Kushner, & Kramer, 2015), for control of gastritis and peptic ulcer and more recently, for the prevention for malaria (Gaillard, Madamet, & Pradines, 2015; Tan, Magill, Parise, & Arguin, 2011). In veterinary medicine, they are mostly used for treating bacterial infectious diseases and also as a growth promoter (Chopra & Roberts, 2001; Jamal, Shareef, & Sajid, 2017; Mathers, Flick, & Cox Jr, 2011). The first group of tetracyclines to be discovered – chlortetracycline,

oxytetracycline, and demethylchlortetracycline were of *Streptomyces* sp. origin. Later semisynthetic ones – methacycline, doxycycline, and minocycline were manufactured (Michalova, Novotna, & Schlegelova, 2004). By 1953, however, isolates of *Shigella dysenteriae* with resistance to the first group of tetracyclines had been discovered (Michalova et al., 2004; Nelson & Levy, 2011a).

Tetracyclines prevent the growth of bacterial cells by binding to the high-affinity binding 30S subunit site of protein 7 (Li et al., 2013; Tritton, 1977) on the bacterial ribosome and thus blocking the binding of an aminoacyl–tRNA. (Brodersen et al., 2000; Connell, Tracz, Nierhaus, & Taylor, 2003; Goldman, Hasan, Hall, Strycharz, & Cooperman, 1983). This disrupts bacterial protein synthesis for cell division. The tetracyclines are highly lipophilic and this enhances their ability to enter bacteria cells to exert their effect (Bryskier, 2005; Nikaido, 2003). Bacteria become resistant to tetracycline by being able to, in an energy dependant manner, flush it out of their cells using an efflux protein located in the lipid bilayer of its cell wall (Li, Livermore, & Nikaido, 1994; Skočková, Cupáková, Karpíšková, & Janštová, 2020; Thaker, Spanogiannopoulos, & Wright, 2010). Bacteria also use ribosomal protection proteins to prevent tetracycline from inserting onto their ribosomes (Velhner & Milanov, 2015) or produce proteins that can modify the chemical structure of tetracycline and prevent them from latching onto their ribosomes (Chopra & Roberts, 2001).

1.4.5 Amphenicols

Chloramphenicol was the first of the amphenicols to be discovered and was isolated from *Streptomyces venezualae* in 1947 as a natural antimicrobial (Aminov, 2017). It contains nitrobenzene and a derivative of dicloroacetic acid (Dowling, 2013). Chloramphenicol is the first natural antimicrobial to be fully synthesized for commercial purposes (Brock, 1961; Ingebrigtsen, Didriksen, Johannessen, Škalko-Basnet, & Holsæter, 2017). It is a broad-spectrum antimicrobial, effective against both Gram-positive and Gram-negative bacteria, *Mycoplasma, Chlamydia, Rickettsia*, and MRSA (Dowling, 2013). Being fat-soluble (Dowling, 2013; Ingebrigtsen et al., 2017), chloramphenicol crosses most physiological barriers in the mammalian body and can be used for treating bacterial infections in the brain, mammary gland, and the placenta (Aminov, 2017). However, due to aplastic

anaemia and bone marrow suppression toxicity during a systemic application, its use has been limited to topical application for eye and ear infections (Ingebrigtsen et al., 2017; Yunis, 1988).

Chloramphenicol is a bacteriostatic antimicrobial that interferes with peptidyl transferase and inhibits peptide bond synthesis at the 50S ribosomal subunit (Tereshchenkov et al., 2018; Weber & DeMoss, 1969; Yunis, 1988). Easily transferred by conjugation, large (> 100 kbp) plasmids carrying the *cml*A gene confers resistance to chloramphenicol (Bischoff, White, Hume, Poole, & Nisbet, 2005; Kehrenberg & Schwarz, 2006). Bacterial resistance to chloramphenicol and its derivative florfenicol is mainly by permeability barriers, mutation of the target site, use of acetyltransferases to inactivate the drug, and use of efflux pumps (Fernández et al., 2012; Schwarz, Kehrenberg, Doublet, & Cloeckaert, 2004; Trivedi, Patil, Shettigar, & Bairwa, 2015).

1.5 Soil microbes and the resistome

The term 'antimicrobial resistome' has been coined to indicate the bacterial ARG pool (Crofts, Gasparrini, & Dantas, 2017; Forsberg et al., 2012; Keen & Montforts, 2011). The presence of both natural and artificial antimicrobials, their metabolites, agrochemicals, industrial chemicals, and other toxins, as well as the variation in physical conditions of the soil due to weather patterns and climatic changes, causes soil microbes to behave differently from microbes in the largely homeostatic environment of living organisms, especially the mammal (Fierer, 2017; Nakatsu, 2007; Prosser, 2015). This is pertinent for the dairy farm soils' microbes, including *E. coli,* as the microbes encounter these wide varieties of synthetic chemicals and their metabolites, (Curutiu, Lazar, & Chifiriuc, 2017; Kalia & Gosal, 2011; Paul, Chakraborty, & Mandal, 2019; Thiele-Bruhn, 2003; Verma, Jaiswal, & Sagar, 2014). Secondly, the variations in physical features like temperature, ultraviolet (UV) and visible light exposure, chemical (water availability, pH, salinity), coupled with the cyclic manner of being ingested (Cooper, Morby, Gunn, & Schneider, 2006; Marles-Wright & Lewis, 2007) into the homeostatic systems of livestock to be later deposited into the farm soil, aggravates the stress on the farm soil microbial population and push them towards adaptations resulting in different phenotypical (Foster, 2007; Walk, Alm, Calhoun, Mladonicky, & Whittam, 2007; Walk et al., 2009) and genetic (Diard et al.,

2010; Landini, Egli, Wolf, & Lacour, 2014) changes. These may result in the development of resistant genes to antimicrobials (Almakki, Jumas-Bilak, Marchandin, & Licznar-Fajardo, 2019; Pal et al., 2017; Ramakrishnan, Venkateswarlu, Sethunathan, & Megharaj, 2019).

1.6 *E. coli:* a model organism for the study of antimicrobial resistance in the environment

From its first discovery by Theodor Escherich (Minodier, 2011), *Escherichia coli* has been found in the gastroenteric system of birds, mammals (Keestra & Bäumler, 2014; Lofton, Morrison, & Leiby, 1962; Mackie, 1997; Nordmann et al., 2012a), reptiles (Cushing, Pinborough, & Stanford, 2011; Gopee, Adesiyun, & Caesar, 2000; Ramos et al., 2019; Wheeler, Hong, Bedon, & Mackie, 2012) and amphibians (Hacioglu & Tosunoglu, 2014; Portis & Coe, 1975). *E. coli* is a Gram-negative, aerobic, facultative anaerobic exhibiting a mutualistic, commensal, and opportunistic pathogenic relationship with its host (Blount, 2015; Luo et al., 2011; Tenaillon, Skurnik, Picard, & Denamur, 2010). *E. coli* is also commonly found in other environmental niches like water bodies (Fujioka, Sian-Denton, Borja, Castro, & Morphew, 1998; Solo-Gabriele, Wolfert, Desmarais, & Palmer, 2000), soil (Bottomley, Angle, & Weaver, 2020; Frankenberger Jr & Arshad, 2020; Fujioka et al., 1998; Solo-Gabriele et al., 2000), and plants (Cooley et al., 2013; Sekizuka et al., 2011).

The vast variation in strain characteristics and adaptations of the *genus* Escherichia has made *E. coli* one of the most studied organisms in biological sciences (Agrimonti, Bottari, Sardaro, & Marmiroli, 2019; Arita, 2004; Frazao, 2018). It has been described as a “classic” organism for studies because of its versatility as a pathogenic or commensal organism (Hornef, 2015; Luo et al., 2011; Nolan, Vaillancourt, Barbieri, & Logue, 2020). It is involved in the physiology and pathophysiology of gastrointestinal, respiratory, urogenital, and other mammalian body systems (Confer & Ayalew, 2013; Heintz & Mair, 2014; Hughes et al., 2010; Macpherson, de Agüero, & Ganal-Vonarburg, 2017; Smith, Fratamico, & Gunther, 2007).

The increase in spread and occurrence of AMR has caused a surge in studies and research into AMR with *E. coli* as the model organism (Blount, 2015; Han & Lee, 2006; Kolisnychenko et al., 2002; Saka

et al., 2005; Stokes et al., 2020; Taj et al., 2014; Vijayendran et al., 2007). *E. coli* has been studied as the organism in whom AMR has been widely monitored (Mellata, 2013; Roca et al., 2015; Skočková et al., 2020). *E. coli* resistant to third-generation cephalosporins, ESBLs, and carbapenems (Chaudhary, 2016) are included in the World Health Organisation's (WHO) list of bacteria that require urgent action to combat its burden on the health and econ*o*my of all nations (Gnanou & Sanders, 2000; Hawser et al., 2009; Kumarasamy et al., 2010; Martel et al., 2000).

E. coli has also been found, in numerous cases, to carry various AMR genes (Burow et al., 2019; EUCAST, 2013; European Food Safety Authority, 2015; Sun, Chen, Jiang, Chen, & Lin, 2019; Tadesse et al., 2012). However, because of the huge variation and characteristics within the species (Agarwal, Srivastava, & Singh, 2012; Blattner et al., 1997), there can arguably never be enough information on them. The present advances in molecular biology together with computerisation, and the widespread use of the internet, has also helped to improved methods of studying, sharing, and storing information about microbes (Cardinale, Joachimiak, & Arkin, 2013; Lee, Esa, Wee, & Soo, 2020; Steurbaut et al., 2010; Willard, Johnson, & Connelly, 1996), and *E. coli* in particular (Kühn, 1985; Rawson et al., 2020; Staropoli & Alon, 2000; Watz et al., 2019).

1.7 *E. coli* and the dairy industry

In the dairy farming industry, the importance of *E. coli* varies from its use as an indicator organism for faecal contamination of milk and other dairy food products (Carlos et al., 2010; Oliver et al., 2020; Salaheen et al., 2019; Stenkamp-Strahm, McConnel, Magzamen, Abdo, & Reynolds, 2018), to an agent of diseases of cows (Bicalho, Machado, Oikonomou, Gilbert, & Bicalho, 2012; Cui, Wang, Lin, et al., 2020; Cui, Wang, Wang, et al., 2020; Edgell, 2020; García, Fox, & Besser, 2010; Low et al., 2005; Sheldon et al., 2010a; Tadesse et al., 2012; Torres Luque et al., 2017), in particular mastitis (Liu et al., 2014) and diseases of calves (Bhullar et al., 2012; Fuenzalida & Ruegg, 2019; Gay, 1965; Jamali, Krylova, & Aïder, 2018; Kolenda, Burdukiewicz, & Schierack, 2015; Rigobelo et al., 2006). In the USA the development of mastitis in a cow costs up to 14.50 USD (LeJeune, Homan, Linz, & Pearl, 2008),

and its treatment range between 224-275 USD/cow, resulting in up to 1.5-2 billion in total cost/year in the USA (Hoffmann, Maculloch, & Batz, 2015).

In the dairy industry, *E. coli* is also implicated in AMR genes (ARGs) carriage and spread (Awosile et al., 2018; Awosile et al., 2020; Barry, 1986; Palmeira & Ferreira, 2020) through the environment by either horizontal or vertical gene transfer (Hammerum & Heuer, 2009; Harrison & Brockhurst, 2012; Oikarainen, Pohjola, Pietola, & Heikinheimo, 2019; Volkova, Lanzas, Lu, & Gröhn, 2012). Apart from bacteria consumed while grazing and drinking, soil microbes may enter the body systems of cows by the teat canal (Derakhshani et al., 2018; Oliver & Mitchell, 1983; Rainard, 2017), as the teat often comes into contact with faecal and soil material which contains bacteria. These could be transmitted to humans through milk as they could be present in the mammary gland before the onset of clinical symptoms (Alharbi et al., 2018; Godziszewska, Pogorzelska-Nowicka, Brodowska, Jagura-Burdzy, & Wierzbicka, 2018; Rainard, 2017).

In the face of the spread of antimicrobial-resistant bacteria, it is important to identify their routes and speed of transmission (Ghosh & Zurek, 2015), to assist with the management of diseases (Chomicz et al., 2014; Stringer, Beard, Miller, & Wakefield, 2002) especially in the presence of widespread antimicrobial resistance (Bergonier et al., 2014). Epidemiologically, the DNA based identification of individual bacterial strains enables the identification of the source(s) of bacteria and the pattern of spread of the bacteria (Winkworth, 2013), which would help to evaluate transmission routes and identify how best to control the spread of these bacteria through the human food chain (Albesharat, Ehrmann, Korakli, Yazaji, & Vogel, 2011; Henriques, Silva, Lemsaddek, Lopes-da-Costa, & Mateus, 2014).

1.8 Antimicrobials in the dairy industry

The main use of antimicrobials in the dairy industry globally is for the treatment and prevention of mastitis (Garcia et al., 2019; Middleton et al., 2014; Sharif & Muhammad, 2009; Tiwari et al., 2013) where bacterium(-ia) is (are) generally the causative agent(s) (Hogeveen et al., 2011; Middleton et

al., 2014; Ruegg, 2017). In the UK, the government has been proactive in promoting the reduction of the use of antimicrobials. In the livestock industry, the UK government managed to reduce the average use of antimicrobials by 19% from 2014 to 2018 at 50 mg/kg across all livestock sectors (Hyde et al., 2017; Mills et al., 2018) with a median of 15.97-20.62 mg/kg in the dairy sector alone (Hyde et al., 2017). Similar to the rest of the world, the NZ dairy industry's use of antimicrobials is mainly for mastitis prevention or cure for non-lactating (dry) (11% of the national total) and lactating cow therapy (1% of the national total) (Agricultural Compounds and Veterinary Medicines Team, 2019).

The majority of the antimicrobials used in the dairy industry are of the intra-mammary route of administration for mastitis control with the occasional use via the injectable form for foot injuries, urogenital infections, or neonatal respiratory and enteric diseases (Garcia et al., 2019; Redding, Bender, & Baker, 2019). In NZ and the rest of the world, the β-lactams of penicillin (cloxacillin with or without ampicillin) remains the main active ingredient (86%) and first-generation cephalosporins (13%) with tetracyclines and aminoglycosides (1%) accounting for the rest of the total antimicrobials used in the dairy industry (Agricultural Compounds and Veterinary Medicines Team, 2019; Fischer, Sjöström, Stiernström, & Emanuelson, 2019; Sawant, Sordillo, & Jayarao, 2005). While most dairy farms in NZ would use antimicrobials based on the advice of a veterinarian, others rely on experience in regards to antimicrobial usage on farms (Fischer et al., 2019; Jones et al., 2015; McDougall, Compton, & Botha, 2017).

There is a good awareness of AMR in bacteria amongst dairy farmers in NZ but the main issues that the farmers concern themselves with are the therapeutic outcomes and the withholding period of the type of antimicrobial used (Jones et al., 2015; McDougall et al., 2017; Mills et al., 2018). Most researchers have indicated poor antimicrobial recording amongst most dairy farmers around the world (González, Steiner, Gassner, & Regula, 2010; Jones et al., 2015; Kayitsinga, Schewe, Contreras, & Erskine, 2017). Most farms would complete an antimicrobial regime at the right dose, and most farmers, instead of veterinarians, administer the antimicrobial regimes (Hyde et al., 2017; Jones et al., 2015; McDougall et al., 2017).

The majority of dairy farms discard milk from cows undergoing antimicrobial treatment within the antimicrobial withholding period, others feed the milk to beef cattle or calves (Brunton, Duncan, Coldham, Snow, & Jones, 2012; Higham et al., 2018). Feeding of milk with traces of antimicrobials as is the case with milk from cows undergoing antimicrobial treatment (De Ruyck & De Ridder, 2007; Han et al., 2015; Meara, 1958; Riediker, Diserens, & Stadler, 2001; Sisodia, Gupta, Dunlop, & Radostits, 1973; Soledad-Rodríguez, Fernández-Hernando, Garcinuño-Martínez, & Durand-Alegría, 2017) has been strongly linked to the development of antimicrobial-resistant strains of bacteria from calves (Aust et al., 2013; Brunton et al., 2012; Langford, Weary, & Fisher, 2003). The appearance of resistant strains of bacteria and other microbes in the environment, especially agricultural lands pose a significant threat not only to persons directly involved in the agricultural industry but also to the population at large due to the transmission of these strains of microbes through the food chain (Allen, 2014; Rebecchi, Pisacane, Callegari, Puglisi, & Morelli, 2015; Wellington et al., 2013). At present, bacteria have developed resistance to most natural and synthetic antimicrobials (Brown & Wright, 2016; D'Costa, McGrann, Hughes, & Wright, 2006). This phenomenon is similar in Europe (Liebana et al., 2012; Tang et al., 2017), America (Allen, Levine, Looft, Bandrick, & Casey, 2013; Michael et al., 2015), and in NZ (Hill, Yung, & Rademaker, 2011; Priest et al., 2017; Ravi, Zhu, Luey, & Young, 2016; Toombs-Ruane et al., 2017). In NZ, the main veterinary uses of antimicrobials (57% of the total of 93 000 kg/year) are for growth promotion and prophylaxis across cattle, poultry, and pig farming (Harrison et al., 2020; Sarmah, Meyer, & Boxall, 2006b).

1.9 Conventional versus organic dairy farming in NZ

In NZ, a predominantly agricultural nation, the number, and intensity of dairy farming have been increasing since the turn of the century (Foote, Joy, & Death, 2015). Dairy farming is one of the major farming practices in NZ and produces 3.5% (17 b NZD) (DairyNZ, 2019) of the gross domestic product of the country whiles contributing 3.3% of total global milk production (Baskaran, Cullen, & Colombo, 2009; Livestock Improvement Corporation Limited & DairyNZ Limited, 2019).

The total number of milking cows in NZ is about 4.9 million from 11 559 herds at an average of 431 cows/herd. Of these, 932 363 cows from 191 herds are found in the Canterbury district of the South Island (DairyNZ, 2019). Dairy farming in NZ is mainly pastoral all year round (Chobtang et al., 2017; Chobtang, Ledgard, McLaren, Zonderland-Thomassen, & Donaghy, 2016; Compton, McDougall, Young, & Bryan, 2014), unlike most other members of the Organisation for Economic and Cooperation Development (OECD) countries. Dairy cows are fed mainly on different cultivars of ryegrass (*Lolium* spp.) and legume foliage of clover (*Trifolium* spp.) (Beukes et al., 2014; Ledgard, Schils, Eriksen, & Luo, 2009; Moot, 2013; Thom, Popay, Waugh, & Minneé, 2014), with the occasional crops of kale (*Brassica oleracea* spp. *acephala*), turnips (*Brassica rapa* subspp.), chicory (*Cichorium intybus*), and fodder beet (*Beta vulgaris* subsp.) during the autumn and winter months (Beukes et al., 2014; Beukes et al., 2012; Doole, 2014). Irrigation on dairy farms is mainly by rivers, streams, creeks, and wells around the farms as well as rainwater collected into tanks and dams (Flemmer & Flemmer, 2007; Martin et al., 2006; Zonderland-Thomassen & Ledgard, 2012). The NZ dairy farming system causes circulation and close interaction between the livestock, the soil microbiome, nutritional elements, other chemicals, their residues, and metabolites (Chobtang et al., 2017; Loganathan et al., 2003; Williams & Haynes, 1990), especially on a conventional dairy farm. The organic dairy farms, however, do not use any chemically formulated nutritional supplements.

The forage-based feed has a lower energy content/kg than grain-based feed, meaning the grass need to be of high quality (Chobtang et al., 2017; Monaghan et al., 2007). Therefore, the use of fertilizers, herbicides, and other agrochemicals is common in the NZ conventional dairy farming industry (Bahmani, Thom, Matthew, Hooper, & Lemaire, 2003; Bouton, 2007; Eerens & Lane, 2004; Ghanizadeh & Harrington, 2019; Laidlaw & Frame, 2013; Rahman, Thompson, & Nicholson, 1990). Some of the common herbicides used on NZ farms such as phenoxy, benzoic acid, dicamba, and the pyridine groups have long residual activity in the soil (Ghanizadeh & Harrington, 2019). Therefore, it is plausible that these substances have some effect on the microbiome of the conventional dairy farm soils. Apart from these, the conventional dairy farming industry uses about 93 000 kg of the active ingredient of commercial antimicrobials annually (Harrison et al., 2020; Ministry for Primary

Industries, 2019; Sarmah et al., 2006a), residues and or metabolites of which get deposited in the soil and or water bodies on the farm. Organic dairy farms, however, do not use agrochemicals, fertilizers, and commercial antimicrobials (Organics Aotearoa New Zealand, 2016).

The total land area of NZ used for dairy farming is about 1.74 million ha. Of this, 2.45% is used for organic dairy farming while the remaining 97.45% is for conventional farming (DairyNZ, 2019; Massey University, 2006; Organics Aotearoa New Zealand, 2016). Organic dairy farming in NZ is attractive because of the perception of ecological friendliness (Mzoughi, 2014; Tuck et al., 2014), the better environmental aspect of nitrogen balance (Anglade et al., 2015; Hansen et al., 2000; Kumar, Patel, Bahadur, & Meena, 2016) and decreased potential of eutrophication due to non-use of fertilizers (Halberg, 2012; Rosati & Aumaitre, 2004; Shah, Bansal, & Singh, 2018). These conditions improve soil and water health (Amin, Karsten, Veith, Beegle, & Kleinman, 2018) and reduce resource intensity and greenhouse gas emissions (Squalli & Adamkiewicz, 2018). This was indicated in long-term studies by Fließbach, Oberholzer, Gunst, & Mäder (2007) and Hartmann, Frey, Mayer, Mäder, & Widmer, (2015).

A four-year NZ study initiated by Massey University, in NZ, compared organic dairy farming to conventional dairy farming and did not find any significant differences in the overall productivity of the two systems (Kelly et al., 2005). However, the cost of the NZ pastoral dairy organic farming was found to be less than the cost of their counterparts in the European Union (EU) and the USA systems (Shadbolt, Kelly, & Holmes, 2005). Although the rules governing organic farming may vary from country to country, in NZ, Australia, and most European countries, an organic farm needs to be accredited by a recognised certifying body (Fouilleux & Loconto, 2017). The European organic definition of holistic production systems based on cultural, biological, and mechanic instead of artificial means has set the standard for organic farming in other parts of the world (Dabbert, Haring, & Zanoli, 2004) and is strictly adhered to by NZ organic dairy farmers. The restricted (or zero) use of antimicrobials, herbicides, pesticides, and synthetic fertilizers in organic farming helps in combating the rise in AMR microbes from the soil because exposure to, and subsequent development of

resistance by microbes to pesticides, leads to the development of resistance to antimicrobials (Kurenbach et al., 2017; Ramakrishnan et al., 2019; Shafiani & Malik, 2003).

1.10 Background to the study

There is a positive correlation between the increased use of an antimicrobial group and the development of resistance against that group of antimicrobials (Korpela et al., 2016; Megraud et al., 2013; Seppälä et al., 1995). This is because the development of resistance by bacteria is mainly due to evolutionary pressure created when bacteria are exposed to sub-doses of antimicrobials, their metabolites, and other chemicals (AliAbadi & Lees, 2000; Andersson & Hughes, 2014; Cassidy, Donnelly, & Tunney, 2010; Grenni, Ancona, & Caracciolo, 2018). This occurs, especially when antimicrobials are used as growth promoters, with the continuous exposure of bacteria to low doses of antimicrobials (Grenni et al., 2018; Van Boeckel et al., 2015). Other authors include the deposition of agrochemicals and their metabolites as well as heavy metals from the industry as factors that cause these evolutionary pressures to the soil microbiome (Coutinho et al., 2013).

The livestock on a dairy farm has constant interaction with the soil microbes due to the amount of soil cattle consume whilst grazing (Cunha & McDowell, 2012; Mayland, Shewmaker, & Bull, 1977), maybe about 0.6% of total dry matter of feed intake (Fries, Marrow, & Snow, 1982) approximately 180-320 kg/animal/year (Healy, 1968). The cattle pass out large amounts of *E. coli* in their faeces (Dargatz et al., 2013; Maule, 2000; Söderlund et al., 2016), which assimilate into the soil and grass, only to be taken up later by the cows. So the cycle continues, with possibilities of transmission to humans (Alharbi et al., 2018; Godziszewska et al., 2018; Muloi et al., 2018; Rainard, 2017). Several studies have shown that pathogenic microbes such as *E. coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, *Mycobacterium paratuberculosis*, several enteric viruses and protozoans, traced to livestock have caused disease outbreaks in human populations (Aijuka & Buys, 2019; European Food Safety Authority, 2015; Gagliardi & Karns, 2000; Goberna et al., 2011; Li, Subbiah, & Dvorak, 2019; Spencer & Guan, 2004). NZ is all too familiar with this phenomenon (Baker, Thornley, Lopez, Garrett,

& Nicol, 2007; Baker, Lopez, Cannon, De Lisle, & Collins, 2006; Jaros et al., 2013; Moore, Drew, Davies, & Rippon, 2017; Nugent, 2011).

The NZ dairy industry is a big player in the global dairy market by contributing 3.3% of the total dairy product consumption in the world (DairyNZ, 2019, 2020). The industry is predominantly of cattle contributing approximately 15.1 billion NZD to the economy as of 2017-18, forecast to rise by 5.5 % to 17.6 billion by the year ending June 2019 (Ministry for Primary Industries, 2020). A total of 7 604 kg, 11% of the national total antimicrobial sold was to the industry in 2017 and the trend had increased in the subsequent years (Ministry for Primary Industries, 2019).

The use of antimicrobials in the conventional dairy farming system compared to the highly restricted to none-usage in the organic dairy farming system in NZ may create conditions that drive the changes in the AMR status of *E. coli* in their soils differently.

1.11 Aims

This study aimed to:

- Adopt a method for antimicrobial susceptibility/resistance analysis that would isolate a diverse range of *E. coli* isolates from the soil of organic and conventional dairy farm soils. Such a method may not show bias towards any particular antimicrobial group by the *E. coli* isolates and analyse the diversity by quadruplex PCR phylogenetic typing (Clermont, Christenson, Denamur, & Gordon, 2013).

- Analyse the phylogeny of the DfSEC isolates to determine whether *E. coli* strains commonly associated with animal species other than bovine may be found in the bovine environment, which may indicate possible transfer from other domestic animals.

- Assess the antimicrobial susceptibility of the dairy farm *E. coli* (DfSEC) isolates from organic and conventional dairy farming systems by the Kirby-Bauer method (Hudzicki, 2009) for the comparison of the effect of the two systems on the antimicrobial susceptibility/resistance of the isolated *E. coli* strains.

- Assess the genetic properties of the DfSEC using whole-genome sequenced (WGS) information analysed by bioinformatics software packages of the DfSEC isolates to compare the effect of the two dairy farming systems on resistance and virulence genes encoded by *E. coli* isolates from the two dairy farming systems.

- Analyse the relatedness of the DfSEC isolates from the four farms located in the same district, within a 20-25 km radius of each other using whole genome multi-locus sequence typing (wgMLST) of *E. coli* (Clermont, Gordon, & Denamur, 2015) for the possibility of transfer of *E. coli* between the farms.

This information would assist in addressing issues that have earlier been raised by different researchers regarding AMR in *E. coli* from the dairy farm environment:

- Antimicrobial resistance threatens the continuous use of antimicrobials to deal with infectious bacteria diseases in medicine, veterinary, and plants.
- The use of antimicrobials in agriculture adds to the development and spread of AMR globally.
- The transfer of antimicrobial-resistant bacteria from agricultural soils to humans is plausible.
- *E. coli* is one of the most important bacteria regarding AMR and this poses problems in the Geraldine district of South Canterbury of the South Island of NZ as it does globally.

Such information could be relied upon by the various AMR controlling stakeholders to make substantiated decisions on the use of antimicrobials in NZ. Data from this study could also be used for future studies on the topic.

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Chapter 2

Isolation and identification of dairy farm soil *E. coli* for antimicrobial resistance studies

2.1 Introduction

Improvements made in culture media and techniques have advanced microbiological research (Bonnet, Lagier, Raoult, & Khelaifia, 2020; Fairfax, Bluth, & Salimnia, 2018; Lagier et al., 2015). The isolation of any particular species of bacteria has traditionally been based on culture techniques (Das et al., 2019; Haruta et al., 2006; Lagier et al., 2015; Lazcka, Campo, & Muñoz, 2007), using an array of specific and/or selective media as a tool to identify and isolate different bacteria (Apostolakos, Mughini-Gras, Fasolato, & Piccirillo, 2020; Hinenoya et al., 2020; Oyeniran et al., 2020; Pagès, Ogier, & Gaudriault, 2020). Since soils contain a very wide variety of bacteria the use of selective media seldom yields a single species (Bonnet et al., 2020; Patel, 2016) and contamination is bound to make such an approach tedious and time-consuming (Ohtsuka et al., 2019). Most researchers have countered this problem by including antimicrobial(s) in the selection media (Bennett, MacPhee, & Betts, 1995; Bonnet et al., 2020; Hustá, Ducatelle, Haesebrouck, Van Immerseel, & Goossens, 2020; Ibekwe & Grieve, 2003; Zadik, Chapman, & Siddons, 1993). However, if the resulting isolated *E. coli* is to be used for an AMR study, the chance of biasing the study by obtaining *E. coli* with specific AMR genes exists.

E. coli isolates from the soil environment are stressed (Evans & Wallenstein, 2012; Manzoni, Schimel, & Porporato, 2012; Moll & Engelberg-Kulka, 2012; Rangel, 2011) and the presence of an energy source may help to repair bacteria strains when culturing them *in vitro* (Aldsworth, Sharman, & Dodd, 1999; Hecker & Völker, 2001; Samson & Cairns, 1977). *Escherichia coli* (EC) broth contains tryptone (0.02 g/mL) and lactose (0.005 g/mL) as an energy source to help the recovery of stressed *E. coli* isolates by contributing to the repair of damaged organelles (Chou & Cheng, 2000; Michel, 2005;

Shafiei, Zarmehrkhorshid, Mounir, Thonart, & Delvigne, 2017). Phosphate buffered saline (PBS) on the other hand, does not contain energy sources and would neither cause the multiplication of cells during incubation nor contribute to the repair of stressed cells.

All *E. coli* strains share the ability to be cultivated on commonly used selective MacConkey agar (MacConkey, 1905; MacConkey, 1900), eosin methylene blue (EMB) (Neidhardt, Bloch, & Smith, 1974), nutrient agar with 4-Methylumbelliferyl ß-D-Glucuronide (NMUG) (Rice, Baird, Eaton, & Clesceri, 2012; Rice, Covert, Johnson, Johnson, & Reasoner, 1995), EC broth (Bordner, 1991; Brenner et al., 1993; Paul, 1980) and tryptone bile X-glucuronide (TBX) agar (Vergine, Salerno, Barca, Berardi, & Pollice, 2017). The use of common culturing methods could safely be employed without bias to obtain a wide range of strains of the organisms, as was shown in studies by Mellmann et al., (2011), and Van Veen, Claas, & Kuijper (2010).

According to Jozefczuk et al. (2010) stressed cells need energy in repairing damaged organelles at the appropriate temperature. Bennett & Lenski, (1993) reported a 40-50% improvement in *E. coli* cell fitness at 42^{o}C but not the multiplication of the cells. Thus a careful manipulation of the incubation media and the temperature of incubation may be employed in culturing a wide variety of strains of a bacteria species from an environment such as the dairy farm soil. The use of standard microbiological culturing techniques was proposed for the isolation of a variety of *E. coli* strains from the dairy farm soils for this study.

This study aims to isolate a wide variety of *E. coli* strains that are not biased toward the possession of AMR genes from the farm soil environment.

2.2 Method development

A triplicate experiment was conducted to compare EC broth (Oxoid, CM0853 Thermo Scientific™, Auckland, NZ) an enrichment selective medium and phosphate-buffered saline (PBS) solution which serves to dislodge bacteria from the soil material as a wash medium (Fatima, Pathak, & Rastogi Verma, 2014; Ghosh & LaPara, 2007; Toljander, Artursson, Paul, Jansson, & Finlay, 2006), at three

different temperatures of 37°C, 42°C, and 44.5°C. This was to determine the appropriate incubation medium and temperature that would result in the optimal isolation of potential *E. coli* from farm soils. Templeton loam soils collected from the Lincoln University conventional dairy farm and the Lincoln University Biological Husbandry Unit (BHU) were used for these experiments. Statistical analysis was done by SigmaPlot 14.0 Kruskal-Wallis ANOVA on ranks.

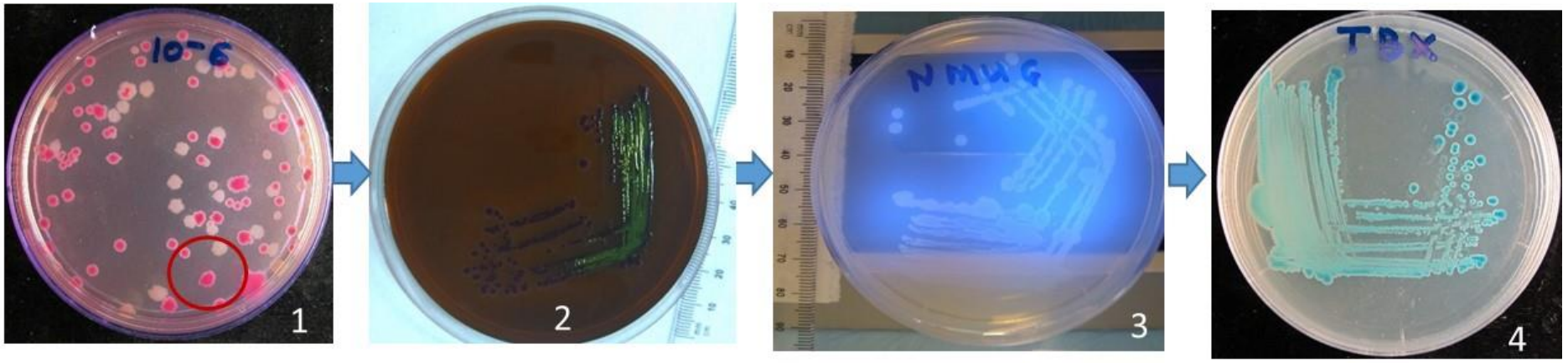
A hand-held auger of 40 mm diameter and 300 mm of depth and a garden trowel was used to collect soil samples including foliage and roots. These were then stored in labelled sterile 1 000 mL screw-capped Shott bottles. Samples were collected from 10 different paddocks at each location. Five random samplings of ~200 g were made per paddock into ~1 000 g of soil/bottle/paddock. Five sampling points with equal distances between them were measured by foot-steps from the paddock gate to the nearest watering trough.

An aseptic technique was used to prevent contamination of samples between different farms. Equipment was washed with tap water, disinfected with 1% Virkon™ solution dried, and sprayed with 70% ethanol. On all farms, during all sampling times, paddocks, where soil samples were taken, had been grazed within the last 24-48 h.

All samples were processed within 24 h of their collection. Twenty-five g of a paddock's composited soil was mixed with 225 mL of EC broth (Oxoid™ CM0853 Thermo Scientific™, Auckland, NZ) in a 2 000 mL sterile stomacher bag. The stomacher bag with the soil was then shaken using a stomacher (Interscience BagMixer®, France) for one min at three stroke/s, to form a soil slurry. The soil slurry was then put into a sterile cotton-plugged 500 mL conical flask and incubated in a shaker-incubator (Thermo Scientific™ MaxQ 4 000, Auckland. NZ) at 37°C, 42°C or 44.5°C at 1 g for 8-12 h (Hakalehto et al., 2010; Patel et al., 2014). A triplicate experiment was conducted at each of the selected temperatures for each ~1 000 g soil sample. One mL of solution was pipetted from the supernatant into 9 mL of sterile PBS solution in 15 mL Eppendorf® tubes and gently vortexed to represent a 10^{-1} dilution. A serial dilution was then prepared from 10^{-1} to the 10^{-6} diluent. From the diluents, 100 µL

of the solution was plated onto MacConkey agar (Oxoid, CM0945 Thermo Scientific™, Auckland, NZ), spread, and incubated at the *E. coli* physiological optimum temperature of 37°C for 24 h in duplicate (Kobayashi, Suehiro, Cach Tuyen, & Suzuki, 2007). A 0.5 nm OD_{600} (SmartSpec® Bio-Rad Laboratories Pty. Ltd, Auckland, NZ) cell suspension of *E. coli* ATCC25922 was prepared and plated on MacConkey agar for comparison, as a positive control. Five colonies, each from a separate agar plate showing a reddish-pink colony with precipitate typical of *E. coli* on the MacConkey agar plates were selected and streak plated onto EMB agar (Oxoid, CM0069 Thermo Scientific™, Auckland, NZ). This was then incubated at 37°C for 24 h. A single typical greenish metallic-sheen-coloured colony similar to the *E. coli* ATCC25922 positive control organism was selected from each plate and streak-plated onto NMUG agar (Oxoid, CM0945 Thermo Scientific™, Auckland, NZ). A colony from the NMUG agar plate was then Gram-stained for the typical *E. coli* morphology and colouration of single pink rod-shaped cells. A colony from the NMUG agar plate showing a bluish fluorescence at 366 nm wavelength of UV was later streaked onto a TBX agar (Oxoid, CM0945 Thermo Scientific™, Auckland, NZ) and incubated at 37°C for 24 h (Figure 2.1). The use of TBX agar further substantiated *E. coli* specificity as well as the ability to sight contaminating colonies without the need of UV lamp as with NMUG agar when -80°C stored samples were cultured. At this stage, cultured colonies that gave a greenish colouration on TBX agar had their identity confirmed by PCR according to Bej, Dicesare, Haff, and Atlas (1991) protocol.

Future isolation procedure may be by spread plating diluents of soil slurry onto TBX agar without the previously mentioned *E. coli* selective agars. This would reduce labour and use of materials. Further the soil slurry may also be divided into different Eppendorf tubes. Number of Eppendort tubes required would be as many as many are required of *E. coli* isolates per composited soil sample. For example in this study, 10 isolates where desired from a composited 1 kg farm soil samples. Thus the soil slurry from the sample could be divided into 10X 15 ml Eppendorf tubes before incubation at 44.5°C in EC broth. This would eliminate of isolating *E. coli* from a single clonal ancestry as an isolate would be selected per tube.



E. coli **strains demonstrate the biochemical and morphological characteristics on selective/indicative media. Tandem use of the selective/indicative media improved the selection and identification of the organism without bias to the *E. coli* strains selected for the study. A serial diluent of dairy farm soil incubated at 37.0 ^{0}C for 24 h in EC broth (25 g in 250 ml) is spread plated on MacConkey agar (1). A typical *E. coli* colony from a diluent with widely spaced colonies is selected by the typical *E. coli* morphology and biochemical representation of reddish pink colony with precipitate, smooth edged and concave rise and transferred by streaking onto EMB agar (2), a colony with the classic metallic green sheen is then streaked onto NMUG agar (3). A colony with fluorescence (366 nm UV wavelength) from NMUG agar is further streaked plated onto a TBX agar (4). A typical *E. coli* colony is selected for PCR species identity confirmation using a *uid*A gene primer pair.**

Figure 2.1 Sequential use of selective/indicative agar media for the isolation of potential dairy farm soil *E. coli* isolates.

The tip of a sterile 200 mL pipette tip was used to pick *E. coli* cells from a separated single colony and put into 20 mL ultrapure DNA/RNA-free water (GIBCO™, Thermo Scientific™, Auckland, NZ) in a 1.5 mL Eppendorf® tube to make a bacterial cell suspension. The cell suspension was then heat-lysed (Brian et al., 1992) at 95°C for 5 min in a heat block (AcuBlock™ Labnet International INC. NJ, USA) and centrifugated (Eppendorf® Minispin® plus, Sigma-Aldrich, Auckland, NZ) at 4 000 g for 5 min, a 2 µL aliquot of the heat lysate bacteria suspension was then used as the DNA template for the *E. coli* specific PCR (Bej, Dicesare, et al., 1991). All DfSEC isolates used for the study were species identity confirmed by PCR. Primers used were: Forward; 5'-AAAACGGCAAGAAAAAGCAG-3' and Reverse; 5'-AC GCGTGGTTACAGTCTTGCG-3' located within the *uid*A structural gene of *E. coli* as outlined by Bej et al. (1991) and also cited by numerous authors (Brasher, DePaola, Jones, & Bej, 1998; Chigor, Ibangha, Chigor, & Titilawo, 2020; Iqbal et al., 1997; Khan et al., 2007; Kibbee, Linklater, & Örmeci, 2013; Maheux et al., 2009; Molina IV & Lowe, 2012; Ntuli, Njage, & Buys, 2017). A 20 µL master mix of 0.2 µL of Taq polymerase, 2 µL of 10X PCR buffer, 2 µL of Q (Bio-Rad Laboratories Pty. Ltd, Auckland, NZ), 2 µL of $MgCL_2$, 0.8 µL each of forward and reverse primers (10 mM), 0.8 µL of dNTPs (10 mM) with DNA template and made up to 20 mL with ultra-pure DNA/RNA-free water. The mixture was placed into a thermocycler (Labnet MultiGene TC 9600G. Sigma-Aldrich, Auckland NZ) for 30 cycles at 94°C denaturing for 1 min and primer annealing at 55°C for 1 min and extension at 72°C for 3 min. The PCR product was visualised following a 2% agarose gel electrophoresis 1X TBE buffer, with 0.07 µL Sybrsafe (Invitrogen®, Auckland, NZ)/mL of gel, run at 90 V for 60 min and visualised with a molecular imager (Gel Doc™ XR+ Bio-Rad Laboratories Pty. Ltd, Auckland, NZ). The farm soil *E. coli* isolates were compared to *E. coli* ATCC25922 for the *E. coli* specific molecular band size of approximately 147 bp (Brasher et al., 1998) referenced to the 1 kb+ molecular marker (Fisher BioReagents™, Thermo Scientific™ Auckland, NZ) (Figure 2.3).

A 50 µL -80°C glycerol stock of 75% brain heart infusion broth (Oxoid, CM1135 Thermo Scientific™, Auckland, NZ): 25% glycerol (Fisher Scientific, Leicester, UK) was prepared from a single greenish colony from the TBX agar plate in a 1.8 mL cryovial (Tarsons CRYOCHILL™ VIAL External threaded Starfoot base-sterile 1.8 mL Kolkata, India) for storage and used for all future analysis.

2.3 Results

Soil samples from the biological husbandry unit and the conventional dairy farm did not show notable differences in the number of potential *E. coli* colonies across the various plates. Incubation with EC broth at 44.5°C resulted in 90% of the colonies that showed the typical *E. coli* colony morphology after tandem culturing on the selected *E. coli* specific agars being confirmed by the *E. coli* specific PCR across all dilution plates from both farming systems (Table 2.1). Incubation in EC broth at 42°C also gave a ratio of an *E. coli*: non-*E*. *coli* colony and cellular morphology of 9:1 (90%). Incubation of the dairy farm soil in PBS at 37°C resulted in a low number of potential *E. coli* isolates compared to non-*E. coli* isolates (> 0.1) ratio. The ratio of potential *E. coli* isolates to that of non-*E. coli* isolates on MacConkey agar after 12 h of incubation, either with PBS solution at 37°C, 42°C or 44.5°C and EC broth at 37°C, 42°C or 44.5°C varied significantly ($P < 0.001$), using the Kruskal-Wallis ANOVA on ranks (SigmaPlot 14.0) (Table 2.1). Incubation in EC broth at 42°C and but not 44.5°C resulted in potential *E. coli* isolates with comparatively larger colonies compared to incubation in PBS at the same temperatures (Figure 2.2). This improved the ease of selecting an isolated colony for further analysis without contaminants. Agar plates that were at lower dilutions than 10^{-6} diluents were over-grown, making it difficult to select individual colonies without contaminants.

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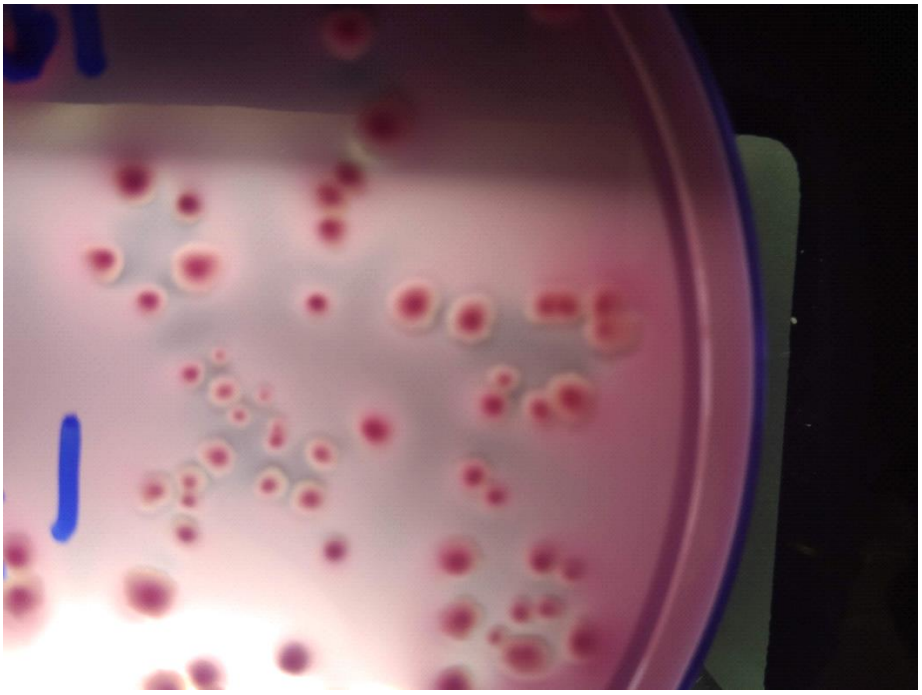
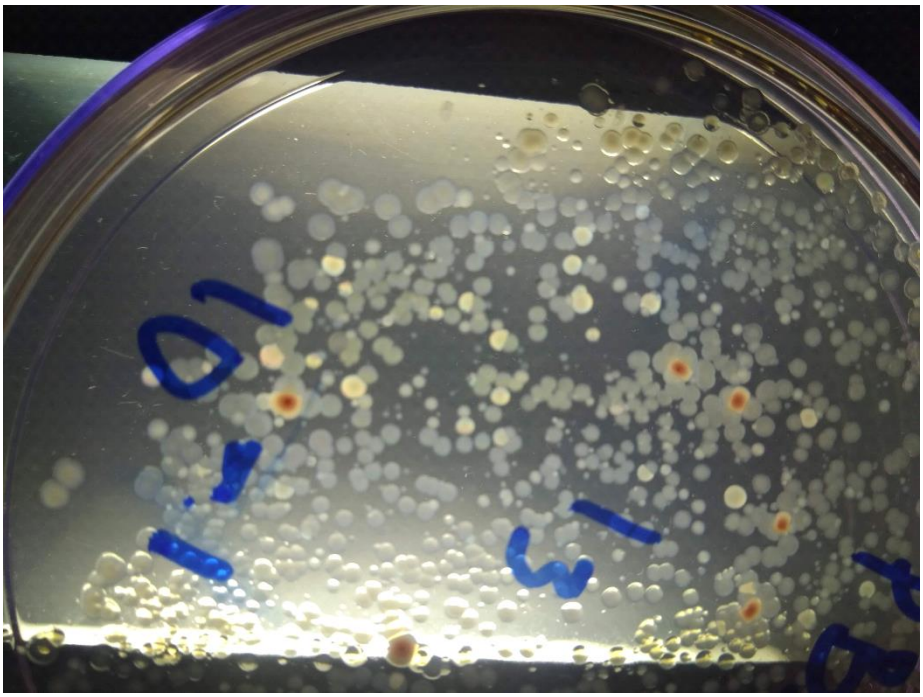


Initial EC broth incubated then cultured on MacConkey agar



Initial PBS incubated then cultured on MacConeky agar

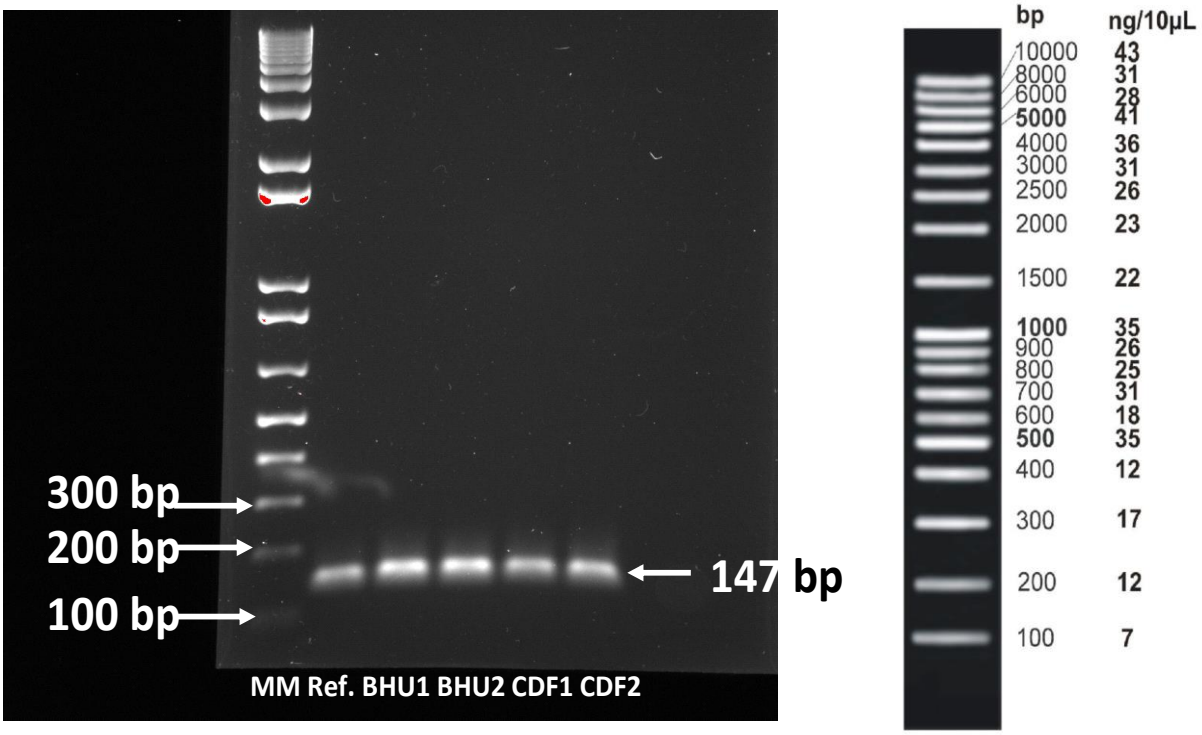
Figure 2.2 Comparison of incubation media

Table 2.1 The mean ratio of potential *E. coli* to non-*E. coli* isolates

	Temperature (°C)		
Incubation medium	37	42	44.5
PBS	0.01± 0.09	0.50± 0.04	0.25± 0.05
EC broth	0.25± 0.09	0.90± 0.09	0.90±0.01

A triplicate isolation experiment from dairy farm soil slurry after 12 h incubation at 37°C, 42°C, and 44.5°C was conducted to determine the most effective media and incubation temperature for best isolation results. At a ratio of 49:1, the colonies that showed typical *E. coli* characteristics on MacConkey agar showed positive characteristics of green-metallic-sheen colonies on EMB and further gave a bluish fluorescence on NMUG under 322 nm UV lamp. All isolates (100%) with greenish colonies on TBX agar, were identified as *E. coli* specific using the *uid*A-based PCR assay (Bej, McCarty, et al., 1991).

In all, 200 *E. coli* isolates with bluish fluorescence at 322 nm UV showed greenish colonies on TBX agar and molecular band ~147 bp on agarose gel electrophoresis using 1xTBE buffer of PCR for the specific *uid*A gene (Bej, Dicesare, et al., 1991) using *E. coli* ATCC25922 as the reference strain (Figure 2.3).



MM=Molecular marker

Ref.= *E. coli* ATCC25922

BHU1, BHU2 = Biological Husbandry Unit isolates 1&2

CDF1, CDF2 = Conventional Dairy Farm isolates 1&2

Figure 2.3. Confirmation of the species identity of presumptive *E. coli* isolates from dairy farm soil by PCR primers for the *E. coli* specific *uid*A gene

2.4 Discussion

As an incubation medium, the EC broth had the potential to cause multiplication of the *E. coli* population in the soil samples which would result in isolates with a common ancestry (Maloy, 1990; Somova, 2020). However, the culturing temperature of 44.5°C, which is outside of the physiological optimum temperature of 37°C for *E. coli* (Ron & Davis, 1971) may have limited such a phenomenon as demonstrated by Lenski & Bennett (1993), Hunke & Betton (2003) and Jozefczuk et al. (2010) in *E. coli* heat-stressed experiments. Ron & Davis (1971) explained that the limited multiplication of *E. coli* cells at temperatures between 40-45°C was due to the heat-sensitive nature of the enzyme homoserine trans-succinylase (in comparison with other essential enzymes) which decreases methionine and thus protein synthesis and slows multiplication.

E. coli in the soil are stressed (Evans & Wallenstein, 2012; Manzoni et al., 2012; Moll & Engelberg-Kulka, 2012; Rangel, 2011) and need energy for repairs (Kamarthapu et al., 2016). PBS does not contain any energy source and did not improve culturing of potentially stressed soil *E. coli* isolates by repair of cell structures or organelles. This would explain the comparatively smaller colonies obtained from PBS incubated soil supernatants.

The results in this study were similar to other studies where culturing methods were used in the isolation of *E. coli* and other microbial species from environmental samples without the use of antimicrobials in the isolation procedure (Ahmed & Shimamoto, 2014; Amoafo, Gooneratne, & On, 2020; Chaudhary et al., 2013; Ishii, Ksoll, Hicks, & Sadowsky, 2006; Zhao et al., 2016). In a method development study Amoafo, Gooneratne, & On (2020) cost-effectively isolated > 200 strains of *Staphylococcus aureus* whose identity were PCR confirmed from conventional and organic dairy farm soils by modification of existing culturing techniques. Apostolakos et al. (2020) used no antimicrobials in the isolation process of *E. coli* from cloacal and carcass samples of broiler chickens from Northern Italy and reported no bias in the isolated *E. coli* profile to antimicrobials and the subsequent genetic study. However, when the authors included cefotaxime antimicrobials in the isolation process of an earlier study (Apostolakos, Mughini-Gras, Fasolato, & Piccirillo, 2019) there

was a bias towards ESBL/AmpC resistant *E. coli* isolates. In another study, Habteselassie et al. (2008) used selected antimicrobials in tandem on soil samples to bias the selection of *E. coli* strains collected in an experiment on the factors affecting the survival and growth of *E. coli* in the soil environment.

In conclusion, this protocol for the isolation of dairy farm soil *E. coli* was sensitive (100% of soil samples examined yielded *E. coli* isolates), appropriate (selective conventional antimicrobial agent not used) and cost-effective approach of obtaining a diverse (belong to different phylogenetic groups, varied antimicrobial susceptibility and growth rate) range of > 200 *E. coli* strains with identity confirmed by PCR (Bej, Dicesare, et al., 1991), for antimicrobial resistance studies.

Chapter 3

Dairy farm soil sampling

3.1 Sampling sites

The Geraldine and Pleasant Point regions are a farming community involved in crops (grains and vegetables), forestry, and animal (dairy cattle, sheep, and deer) farming. The region comprises the counties of Geraldine, Levels, Mackenzie, and Waimate. It is bounded in the north by the Rangitata River, Forest Creek, and part of the Two Thumb mountain range, in the west by the crest of the Southern Alps, in the south by the Waitaki River, and in the east by the Pacific Ocean. Of the total area of 137 600 km^2, 86% is farmed. The soils of this Canterbury region are silty sandy loams, formed mainly from greywacke alluvium. The soils have variation in-depth as they are underlined with gravel and boulders. The soil may be stony throughout its profile or maybe 20 to 100 mm of silt or sandy loams above the shingle (Landcare Research, Soil Map online https://smap.landcareresearch.co.nz/). The vegetation type on most dairy farm fields (paddocks in NZ) is ryegrass (*Lolium* sp.) with white and red clover (*Trifolium repens* and *Trifolium pratense*) swards.

Within the Geraldine and Pleasant Point catchment of the South Island of NZ, are two organic dairy farms, each of which has a conventional dairy farm within 5-10 km distance. The Clearwaters (CW) organic dairy farm (GPS: 44°15'53.8"S 171°10'11.2"E) and Peel Forest (PF) conventional dairy farm (GPS: 44°00'50.4"S 171°16'26.4"E) are slightly closer together, about ~5 km apart. While the Totara Valley (TL) organic dairy farm (GPS: 44°14'17.5"S 171°04'02.1"E) and Mill Road (MRD) conventional dairy farm (GPS: 44°16'23.9"S 171°10'12.7"E) are also about 5 km apart. All four farms are located within a ~20-25 km radius.

This presented a unique opportunity to study the effect that dairy farming husbandry systems with the use of antimicrobials, agrochemicals, and chemical fertilizers have on the AMR profile of *E. coli* isolated from their soils. These farms are similar in size, having 600-800 milking cows, stock concentration (cows/ha), with each husbandry stocking breeds of over 90% Holstein Friesian,

vegetation, and soil type. All four farms practice similar husbandry of twice a day milking, dates and methods of drying off, calving schedules, and breeding regimes. All farms have been in operation for over a decade, enough time for the type of husbandry system to have long-term effects on the soils and the microbes in the soils.

Based on the results of the method development conducted in the preliminary study (Chapter 2), the same sampling and isolation methods were used in this main study.

3.2 Sampling times

The sampling of soils from the four dairy farms was conducted at four time-points with the probability of variation in *E. coli* characteristics being shaped by seasonal variations of soil moisture and temperature (Coneyworth et al., 2020; Mattiello, 2016). This is more so in New Zealand where dairy farming is mainly pasture-based (Cuttance et al., 2017; Li, Snow, & Holzworth, 2011) and geographically exposed to relatively high UV index (Bornman et al., 2015; Moran et al., 2004). To this effect, efforts were made to sample at different times of the year when different husbandry practices were undertaken in the New Zealand dairy industry and for consecutive years. Unfortunately, due to logistical problems, sampling during the summer months was missed (Table 3.1).

Table 3.1 Dairy farm soil sampling calendar

Sampling order	Season	Month	dairy farm practice	year
first soil sampling	spring	October	start of calving to start of mating	2017
second soil sampling	spring	October	start of calving to start of mating	2018
third soil sampling	autumn	March	milking/grass pasture management	2018
fourth sampling	winter	June	milking/crop pasture management	2018

3.3 Results

For each sampling time, > 200 dairy farm soil *E. coli* (DfSEC) isolates of at least 50 isolates per farm whose identity was confirmed by PCR (Bej, Dicesare, et al., 1991) were isolated from the four farms and stored in a 25% glycerol stock in BHI (Chapter 2) at a temperature of -80°C for future analysis. Such that in total, for this study, 814 DfSEC were isolated over the four-time sampling periods (Table A1.1).

3.4 Discussion

In this study, the choice of the experimental site enabled the elimination of bias that may have resulted from differences in climatic/weather conditions, soil, and vegetation types. Further, the location of Geraldine at the foothills of the snow-capped Southern Alps meant the farms sourced water from a location that was comparatively uncontaminated by human activity. This would arguably mean the *E. coli* isolates in the soil would only be affected by the practices on the farm, giving more meaning to the results of the experiment. The selected conventional dairy farms had an organic dairy farm located within a 5-10 km radius and this set up a scenario for comparison of the effect of the husbandry practices on the farms, limiting other confounding factors. However, cross-contamination of the farms by wildlife (Nugent et al., 2011), shared contractors (Kerr & Layton, 1983; Rijswijk & Brazendale, 2017), and possibly waterways (Dymond, Serezat, Ausseil, & Muirhead, 2016; Monaghan, De Klein, & Muirhead, 2008) could not be ruled out. The soil sampling adopted in this study, from the paddock gate to the nearest water trough would have promoted the chances of isolating *E. coli* strains that would have had interaction with the majority of cows on the farm.

Chapter 4

Antimicrobial susceptibility testing of dairy farm soil *E. coli* isolates

4.1 Introduction

In an antimicrobial resistance study, antimicrobial susceptibility testing (AST) is used as an indicator of phenotype expression of resistance/susceptibility of a bacterium to a particular antimicrobial or class of antimicrobials (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2019; Peng et al., 2017; Threlfall, Fisher, Ward, Tschape, & Gerner-Smidt, 1999). In medicine, AST helps to improve the precision in antimicrobial therapy to help reduce the burden that bacterial diseases cause, both to humans and in agriculture by reducing the unsubstantiated use of antimicrobials (Dietvorst, Vilaplana, Uria, Marco, & Muñoz-Berbel, 2020; Mohan et al., 2013). AST provides specific information for therapeutics and other antimicrobial usages (Dietvorst et al., 2020; Vineetha, Vignesh, & Sridhar, 2015).

Frequently, AST is based on a modified version of the protocol formulated and standardised by W. M. Kirby and his colleague, A. W. Bauer in 1960, working at the University of Washington, School of Medicine (Bauer, Kirby, Sherris, & Turck, 1966). This was adopted by The WHO as the standard guideline for AST globally in the 1960s (Hudzicki, 2009). Antimicrobial susceptibility testing (AST) may be done in a liquid or solid medium (Choi et al., 2013; Clinical Laboratory Standards Institute (CSLI), 2019; EUCAST, 2019; Humphries et al., 2018). With the solid agar medium (Kirby-Bauer method), a known concentration of an antimicrobial disc (~6 mm in diameter) is put on a known concentration of bacteria evenly spread over an agar medium (Hudzicki, 2009). After the incubation at optimal conditions, the area of inhibition of bacteria growth around the disc indicates the susceptibility/resistance of the bacteria to the antimicrobial used (CLSI, 2019; EUCAST, 2019; Humphries et al., 2018).

4.2 Materials and methods

Mueller-Hinton (MH) agar plates (Oxoid CM0337 Thermo Scientific™, Auckland, NZ) were prepared according to the manufacturer's instructions. Briefly, a solution of the agar base was sterilized by autoclaving at 121°C for 15 min and poured into a sterile Petri dish to a depth of 4 mm, dried, and stored in plastic bags at 4°C until use (Cockerill, 2011). Plates of MH agar used at all times were freshly prepared and not more than five days old.

A single colony of the dairy farm soil isolated *E. coli* growing on a TBX agar plate at 24 h was used to inoculate 5 mL of 0.1 M PBS solution using a sterile microbiological loop and gently vortexed for 10 s. The turbidity of the bacteria suspension was compared to the turbidity of 0.5 McFarland standard solution (0.5 mL of 0.048 M $BaCl_2$ to 99.5 mL of 0.18 M H_2SO_4) and measured by a photo spectrometer (SmartSpec3000™ Bio-Rad Laboratories Pty. Ltd, Auckland, NZ) to be between 0.08-0.1 nm OD_{600} (EUCAST, 2013).

Table 4.1 List of antimicrobials and their concentrations used for the study

Antimicrobial	Concentration µg	Symbol
cefoxitin	30	FOX30
cefpodoxime	10	CPD10
chloramphenicol	30	C30
ciprofloxacin	30	CIP30
gentamicin	10	CN10
meropenem	10	Mem10
nalidixic acid	30	Na30
tetracycline	30	Te30

A fresh sterile cotton bud was immersed in the bacteria suspension and pressed against the bottle container to remove the excess bacterial suspension and then used to make an initial mat spread onto the MH agar plate. This was repeated after turning the plate at 90° to obtain a uniform spread

of bacteria on the agar surface. Antimicrobial discs (Oxoid™ Thermo Scientific™, Auckland, NZ) for selected antimicrobials (Table 4.1) stored in a desiccant at 4°C were placed on the agar and firmly pressed using forceps sterilised by dipping in 95% ethanol and flamed.

The agar plates were first held with the right-side-up (lid-side-up) for about 5 min and later inverted (within 15 min of plating) and incubated at 37°C for 24 h (Andrews & Testing, 2001). The inhibition zone diameters (mm) at the point of inhibition were measured using a ruler and interpreted into resistant (R), intermediate (I) and susceptible (S) reactions according to the European Committee on Antimicrobial Susceptibility Testing EUCAST (2015) breakpoints for the disc diffusion method of antimicrobial susceptibility testing (Table A1.1). However, for nalidixic acid, the breakpoint of inhibition zone used was as indicated by the British Society for Antimicrobial Chemotherapy (BSAC) methods for AST (Wootton, 2014) as the EUCAST (2015) document had 'not applicatble'(N/A) for nalidixic acid on *E. coli*. Inhibition zones of the dairy farm soil isolated *E. coli* were compared to reference *E. coli* NCTC12241 and *E. coli* ATCC25922 in all cases as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2015). Records of the antimicrobial profile of at least 50 PCR confirmed *E. coli* (Bej, Dicesare, et al., 1991) from each farm for each of the four sampling times (spring 2017, spring, autumn, and winter of 2018) were recorded on a Microsoft Excel spreadsheet and data was analysed using the fit binary regression model in Minitab19 statistical software at 95% confidence interval (CI).

The criteria for the selection of the group of antimicrobials (Table 4.1) for this study was based on the following rationale.

a) cefpodoxime as a third-generation cephalosporin and ESBL (Shankar & Balasubramanium, 2014)
b) chloramphenicol, ciprofloxacin, gentamicin, meropenem, and tetracycline as listed by the World Health Organisation (WHO) essential medicine (Organization, 2019);

c) cefoxitin and meropenem as the yardstick for a potential ESBL resistant organism, as recommended by EUCAST for antimicrobial susceptibility testing (EUCAST, 2019); and

d) nalidixic acid as a synthetic antimicrobial, resistance to which may be as the result of human activity only (Kyzioł, Khan, Sebastian, & Kyzioł, 2020; Michael, Dominey-Howes, & Labbate, 2014).

4.3 Results

The AST for the four farms, over four sampling times against eight different antimicrobials with a total of 7 224 tests (Table A1.1) showed that 3.7% of the isolates tested were resistant to at least one of the eight selected antimicrobials. A summary of DfSEC isolates showing intermediate-resistant/resistant to the selected antimicrobials is provided in Table 4.2

Table 4.2 Summary of DfSEC showing intermediate-resistance/resistance to selected antimicrobials

		Antimicrobial								
	farm/season/year	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Total
Organic	CWS17	11	0	15	0	4	0	12	0	42
	CWS18	0	1	2	1	0	0	2	0	6
	CWA18	2	0	2	0	0	0	2	0	6
	CWW18	0	0	0	0	2	0	1	0	3
	TLS17	1	0	10	0	0	0	18	0	29
	TLS18	0	0	6	0	1	0	1	0	8
	TLA18	0	0	3	4	1	0	11	0	19
	TLW18	0	0	0	0	0	0	3	1	4
Conventional	MRDS17	1	6	4	0	8	0	5	0	24
	MRDS18	1	1	3	0	1	0	2	4	12
	MRDA18	0	0	2	0	0	0	8	0	10
	MRDW18	0	0	5	1	0	0	11	0	17
	PFS17	9	4	5	10	3	0	2	2	35
	PFS18	6	0	2	0	0	0	2	0	10
	PFA18	5	0	4	0	0	0	10	0	19
	PFW18	2	0	3	0	0	0	9	2	16
	Total	**38**	**12**	**66**	**16**	**20**	**0**	**99**	**9**	**260**

Statistical analysis showed a significant difference (P < 0.0001) between the eight different antimicrobials used and the sampling time point of the DfSEC isolates. When the different farming systems were compared but similar locations, the Peel Forest conventional dairy farm was 0. 65 (odds ratio) times more likely to be resistant compared to the closely located Clearwaters organic dairy farm, but the DfSEC isolates from the Mill Road conventional dairy farm were 0.58 (odds ratio) less likely to be resistant compared to the closely located Totara Valley organic dairy farm. When similar farming systems but different locations were compared, the Peel Forest conventional dairy farm showed less chance of isolates being resistant compared to the Mill Road conventional farm with an odds ratio of 1.22. The Totara Valley organic farm had a higher chance of the DfSEC isolates being resistant compared to the Clearwaters organic farm with an odds ratio of 0.78 (Table 4.3).

Table 4.3 Comparison between the antimicrobial profile of resistant dairy farm soil *E. coli* isolates by farming systems and geographical location over four seasons of sampling. At 95% CI

Conventional farm		**Organic farm**	**Odds ratio: CI**
Mill Road		Totara Valley	0.58: 1.06, 2.09
Mill Road		Clearwaters	0.58: 0.36, 0.78
	vs		
Peel Forest		Clearwaters	0.65: 0.43, 0.95
Peel Forest		Totara Valley	1.22: 0.86, 1.73
	Conventional farms		
Peel Forest	vs	Mill Road	1.22: 0.87, 1.70
	Organic farms		
Totara Valley	vs	Clearwaters	0.78: 0.53, 1.16

Combined data from the two organic farms were compared to combined data from the two conventional farms (Table 4.4) and the spring of 2017 data showed a significant difference (P < 0.01),

with a higher prevalence of resistance in the organic farms compared to the conventional farms (odds ratio 1.72). However, in the spring and winter of 2018, the prevalence of resistance was significantly higher ($P < 0.0001$) in the conventional farms compared to the organic farms (odds ratio of 0.20, 0.08) respectively. In the autumn of 2018, there was no significant difference between the two farming systems regarding the prevalence of resistance ($P = 0.29$) but the organic farms showed 0.74 less chance of showing resistance compared to their conventional counterparts (0.74 odds ratio of resistance). In this study, a comparison of the percentage resistance of the 814 DfSEC to the eight selected antimicrobials was compared between the organic farm soil isolates and their conventional farm counterparts at each sampling-time point. The P-value and odds ratio of the comparison was assessed at 95% CI. Overall, DfSEC isolates from the organic dairy farms showed a lower prevalence of resistance to the antimicrobials tested, compared to their counterparts from the conventional farms. (Table 4.4).

Table 4.4 Summary of resistance against select antimicrobials demonstrated by isolated dairy farm soil *E. coli*

Season/year. Organic (n=2) vs Conventional (n=2) dairy farms. Confidence interval (CI) of 95%

		% of DfSEC resistant to selected antimicrobials between conventional and organic dairy farms									
Sampling–time point	**Farming system**	FOX30	CPD10	CIP10	C30	CN10	Mem10	Na30	Te30	P-value	Odds ratio: CI
Spring 2017	organic	36.9	9.6	0.0	0.0	0.0	12.3	0.0	11.4	< 0.01	1.72: 1.14, 2.60
	conventional	21.0	5.4	0.0	0.0	2.0	26.9	2.9	6.7		
Spring 2018	organic	7.7	0.0	0.0	0.0	0.9	0.0	0.9	0.0	< 0.0001	0.28: 0.15, 0.52
	conventional	12.9	0.9	0.0	3.9	0.0	12.0	0.9	7.2		
Autumn 2018	organic	15.4	4.8	0.0	2.9	3.8	2.0	0.0	1.9	0.29	0.74: 0.42, 1.30
	conventional	13.1	0.0	0.0	0.0	0.0	3.0	0.0	4.3		
Winter 2018	organic	5.1	0.0	0.0	1.0	0.0	2.0	0.0	0.0	< 0.0001	0.09: 0.03, 0.25
	conventional	20.5	5.9	0.0	7.0	3.0	0.9	1.0	1.0		

FOX30=cefoxitin 30 µg/disc: CPD10=cefpodoxime 10 µg/disc: CIP10=ciprofloxacin 10 µg/disc: C30=chloramphenicol 30 µg/disc

CN10=gentamicin 10 µg/disc: Mem10= meropenem 10 µg/disc: Na30=nalidixic acid 30 µg/disc: Te30= tetracycline 30 µg/disc

4.4 Discussion

In this study, the use of the Kirby-Bauer disc diffusion method for AST enabled the differentiation of DfSEC into resistant and susceptible isolates to a range of eight different antimicrobials. Since its adaptation by the WHO as a standard method for AST the Kirby-Bauer disc diffusion method has been widely used and has been compared positively to other phenotypic testing methods (Bhatt, Tandel, Shete, & Rathi, 2015; Bukhari, Ahmed, & Zia, 2011; Joseph et al., 2011; Murray, Niles, & Heeren, 1987; Vyas, Sharma, Kumar, Kumar, & Mehra, 2015). The choice of method for AST depends on the study organism (wild-type versus non-wild-type) and the antimicrobial of choice with regards to the availability of breakpoint data (CLSI, 2019; EUCAST, 2019). In this study, there was available data from the two frequently used guidelines on the antimicrobials used as well as the study organism of DfSEC. Although for nalidixic acid, the reference breakpoint used was for *E. coli* obtained from urinary tract infection studies EUCAST (2015). The disc diffusion method is easy to use (Bukhari et al., 2011) and available to the standard microbiological laboratory (Vyas et al., 2015). Besides, further testing using the phenotype screening kit MAST™ of combined double-disc synergy test (CDDST) (Chapter 5) was done to confirm the disc diffusion method used earlier in the study (Stuart & Leverstein-Van Hall, 2010).

The antimicrobial profile of the DfSEC isolates from the conventional dairy farms where a significant amount of antimicrobials, herbicides, pesticides, and inorganic nitrogen-based fertilizers are frequently used indicated a higher percentage of resistant DfSEC isolates compared to isolates from the organic dairy farms during three of the four sampling-time points. However, the organic dairy farms were not devoid of antimicrobial-resistant strains as the spring 2017 sampling indicated a significantly higher prevalence of resistant isolates from the organic system compared to the conventional. In NZ, conventional dairy farming used about 11% (10 230 kg of antimicrobials) of the national antimicrobial usage in 2017 (Ministry for Primary Industries, 2019) and this trend has been increasing (Ministry for Primary Industries, 2019). Since about 30-80% of antimicrobial used is excreted whole or as metabolites (Manzetti & Ghisi, 2014; Schallenberg & Armstrong, 2004;

Srinivasan, Sarmah, & Manley-Harris, 2014), a significant amount of antimicrobials is excreted into the conventional dairy farm soils and impact the soils microbiome's AMR status (Schallenberg & Armstrong, 2004), as opposed to organic dairy farming. For instance, a 2014-15 study in five different regions in New Zealand including North Canterbury indicated ~4.8 mg of active ingredient/population correction unit (PCU, defined as the mass of active ingredient divided by total biomass) to ~684 000 cows. This is considered low compared to international standards (Bryan & Hea, 2017).

It has been argued and confirmed by some authors that the use of antimicrobials and other agrochemicals in conventional dairy and other agricultural husbandry systems increases the amount of AMR bacteria and ARGs in the bacteria compared to the limited/non-usage of these chemicals on organic farms (Awad et al., 2014; Österberg et al., 2016; Schwaiger, Schmied, & Bauer, 2010). However, a study in four different European countries comparing the AMR from pig faecal samples from organic and conventional farms found no difference between the AMR of isolated microbes from the farming systems (Gerzova et al., 2015). Similarly, in this study, overall, the antimicrobial and other agrochemical usages by the conventional dairy farms in the Geraldine area of South Canterbury may not have affected the antimicrobial status of *E. coli* isolates in the farms' soil significantly, compared to the limited/non-usage of antimicrobials and other agrochemicals by the organic dairy farms. This may also be attributed to the pastoral type of farming by both systems where the cows are held on a vast area of land all year (Hancock, 1950) round and not in the small defined area as in intensive dairy farming systems in other organisations for economic co-operation and development (OECD) countries (Hancock, 1950; Hillerton, Irvine, Bryan, Scott, & Merchant, 2017; Lewthwaite, 1964). Secondly, the Geraldine area is a rural community of 2 301 in 2013 (http://infoshare.stats.govt.nz/Census/2013) and 138 km from the nearest industrialised city of Christchurch. This would limit industrial and domestic waste and chemicals that may seep into the agricultural lands during rainwater run-offs and flooding times. Further, being located at the foothills of the snow-capped Southern Alps means the farms' source of water from rivers, streams, creeks, and wells are comparatively uncontaminated with chemicals that would impact the microbial population in their soils in comparison to that in other parts of the country.

The definition of AMR varies depending on whether the term is used clinically, epidemiologically, or gene-centrically with the comparison between wild-type and non-wild-type strains (Martínez, Coque, & Baquero, 2015). The definition of antimicrobials (Chapter 1) indicates that antimicrobials in the dairy farms soil could be from the soil's natural microbiome as soil bacteria release antimicrobials into their environment to protect themselves from other bacteria (Martinez, 2009; Martínez, 2008; Martínez, 2012; Nesme & Simonet, 2015; Raaijmakers & Mazzola, 2012; Wellington et al., 2013). Thus in the organic or conventional dairy farms soil, the existence of antimicrobial agents and their metabolites irrespective of the impact of human activities cannot be ruled out (Berendonk et al., 2015; Martínez et al., 2015). For instance analysis of soil collected from 12 organic farms in Nebraska, the USA between May and June of 2013 found 93% of the soil samples contained sulphonamides and tetracycline resistance genes (Cadena et al., 2018).

However, the effect of an antimicrobial on the metabolism of a bacteria depends on the adequate concentration of the antimicrobial reaching the site of its action on the target microbe (Lietman, 1986; Lobritz et al., 2015; Maier et al., 2018). As such, from the conventional dairy farm as opposed to the organic dairy farm, it would be expected that adequate concentrations of antimicrobials and their metabolites, as well as those of other agrochemicals, affect the soil's microbiome to a greater extent.

In this study, the soil samples from which *E. coli* isolates were isolated for their antimicrobial profiling were not screened for the presence of antimicrobials or their metabolites which, as previously argued, may affect the resistance/susceptibility status of the *E. coli* isolated from them (Li, 2014; Thiele-Bruhn, 2003). However, since antimicrobials in the environment, especially the farm soil environment are ubiquitous (Martínez et al., 2015), it would be impossible to determine how much of it is due to human activities or otherwise unless time and quantity of antimicrobials from human activity deposited in that environment over a period were determined (Berendonk et al., 2015).

Chapter 5

Confirmatory phenotypic screening for dairy farm soil *E. coli* showing resistance to selected antimicrobials

5.1 Introduction

Confirmatory phenotype screening of an organism for its potential to produce a resistant enzyme is indicated when reduced susceptibility to a group of antimicrobials is detected in a routine AST (Thomson, 2010). For the broad-spectrum β-lactam groups of carbapenems, monobactams, and cephalosporins the chosen antimicrobials are cefoxitin, cefpodoxime, and imipenem, respectively (Al-Sarawi, Jha, Baker-Austin, Al-Sarawi, & Lyons, 2018; Earnshaw et al., 2013; EUCAST, 2013). The underpinning logic for this procedure, according to the Public Health England (2016) and Stuart & Leverstein-Van Hall (2010), is the need to effectively identify bacterial strains that produce the various classes of β-lactamases while differentiating them from strains whose resistance are conferred, either solely or in combination with other resistance mechanisms such as hyper-expression of the efflux pump mechanism. This is because, clinically, antimicrobial resistance by the release of an antimicrobial degrading enzyme (agent) may be solved by the use of inhibitors like avibactam, vaborbactam, relebactam (Wong & van Duin, 2017), sulbactam, tazobactam, or clavulanic acid (Papp-Wallace & Bonomo, 2016) against the degrading enzyme, but not when the microorganism hyper-expresses the efflux pump or reduced permeability mechanism for antimicrobial resistance. For instance, some Enterobacteriaceae including *E. coli* and *Pseudomonas auroginosa* produce β-lactam hydrolysing enzymes, may hyper-express the efflux pump mechanism (Livermore, 2001), or change the cell permeability of an antimicrobial by modifications of their outer membrane porin proteins (Doumith, Ellington, Livermore, & Woodford, 2009).

A confirmatory phenotype screening procedure tests an antimicrobial, and a known inhibitor(s) of the class of β-lactamase (Public Health England, 2016). Both double-disc synergy test (DDST) consists

of an antimicrobial disc only and the disc with only the inhibitor (Giske et al., 2011; Giske et al., 2013; Khosravi, Hoveizavi, & Mehdinejad, 2013; Pailhoriès et al., 2014) and the combined double-disc synergy test (CDDST), antimicrobial disc only and a disc with antimicrobial combined with enzyme inhibitor are widely used (Kumar, Arora, Mishra, & Dogra, 2018; Numanović et al., 2013; Pandya, Prajapati, Mehta, Kikani, & Joshi, 2011). The inhibition zone shown by the test organism to the antimicrobial is compared to the inhibition zone against the β-lactamase inhibitor. All the tests are based on the use of enzyme inhibitors such as boronic acid, cloxacillin, clavulanic acid, or ethylenediaminetetraacetic acid (EDTA) against positive and negative control organisms. When the difference in the distance as indicated by the manufacture of the kit being used is greater than the specified distance (mm) a judgment is made as to whether the test organism produces the β-lactamase enzyme against the antimicrobial being tested for, or not.

Presently, screening test kits available for confirmatory phenotypic screening are Oxoid (Dardilly, France) (Garrec, Drieux-Rouzet, Golmard, Jarlier, & Robert, 2011), Mast CO. (Liverpool, UK) (Nourrisson et al., 2015; Safari, Mozaffari Nejad, Bahador, Jafari, & Alikhani, 2015), direct-on-target microdroplet growth assay (DOT-MGA) (Correa-Martínez, Idelevich, Sparbier, Kostrzewa, & Becker, 2019) and the βLACTAM method (El-Jade et al., 2018; Poirel, Fernández, & Nordmann, 2016). However, the βLACTAM method is based on a colour change of the hydrolysed β-lactam antimicrobial from yellow to red, of a carboxy-propyloxyimino-containing compound HMRZ-86 (Hanaki et al., 2007; Hanaki, Kubo, Nakano, Kurihara, & Sunagawa, 2004; Morrison & Siu, 2000). The level of sensitivity and specificity of such chromogenic tests depend on the type of resistance mechanism, the species of bacteria, and the level and type of resistant expression as shown in a study by Renvoisé et al. (2013).

5.2 Materials and methods

A total of 200 isolates that showed resistance to FOX30 and Mem10 of the carbapenem antimicrobial group and/or CPD10 of the cephalosporin group of antimicrobials, zero inhibition zone (6 mm, disc diameter) to Te30, and > 15 mm diameter against C30 were selected as potential carbapenemase,

AmpC, and ESβL(s) producers and phenotypically screened. For the detection of AmpC and/or ESBL enzyme-producing *E. coli* isolates, MASTDISCS® Combi AmpC and ESBL Detection Discs D68C commercial kit (MAST™ Group Ltd, Liverpool, UK) were used. The test kit was made up of four different discs (Table 5.1). For the detection of carbapenemases class A [*Klebsiella pneumoniae* carbapenemase (KPC)] type, class B Metallo-β-lactamases (MBLs), and class D, oxacillinases-48-like (OXA-48) enzyme production (Doyle et al., 2012), the combined double-disc synergy test (CDDST) with MASTDISCS® combi Carba plus D73C (MAST™ Group Ltd, Liverpool, UK) kits were used (Table 5.2).

Table 5.1. MASTDISCS®kit (D68C) for phenotype screening for potential AmpC and extended-spectrum β-lactamase-producing dairy farm soil *E. coli* isolates

Disc label	Disc content
A	cefpodoxime 10 µg
B	cefpodoxime 10 µg+ESβL inhibitor
C	cefpodoxime 10 µg+AmpC inhibitor
D	cefpodoxime 10 µg+ESβL+AmpC inhibitor

All discs were stored at 4°C and used according to the manufacturer's instructions and no expired discs were used. *E. coli* NCTC13351 was used as ESβL positive and *E. coli* ATCC25922 as ESBL, KPC, MBL, and OXA-48 negative controls.

Table 5.2. MASTDISCS® (D73C) combi Carba plus for phenotype screening of potential carbapenemase-producing dairy farm soil *E. coli* isolates

Disc label	Disc content
A*	penem
B*	penem+MBL inhibitor
C*	penem+KPC inhibitor
D*	penem+AmpC inhibitor
E*	temocillin+MBL inhibitor

Briefly, a single *E. coli* colony from a TBX agar plate not more than 24 h old was used. The cell concentration, method of spread, and disc placement were carried out similarly to the disc diffusion method of antimicrobial susceptibility testing procedure described previously (Chapter 4). All four discs (A, B, C, and D) were placed on an agar plate with sufficient spacing between them so as not to fuse inhibition zones. Weekly quality control of disc performance was conducted using a negative control *E. coli* ATCC25922 during the phenotype screening periods. The interpretation of the test results was made according to the manufacturer's instructions as follows:

For AmpC and ESBL detection discs D68C (MAST™ Group Ltd, Liverpool, UK) (Figure 5.1).

All inhibition zones differ by ≤ 2 mm No ESBL or AmpC activity.

Inhibition zone of ≥ 5 mm between antimicrobial-only disc and any of the antimicrobials combined with inhibitor, the organism is positive for the release of a β-lactamase enzyme (ESBL or AmpC activity).

When differences between discs are ≤ 2 mm the organism is negative for the release of a β-lactamase.

D–B ≥ 5 mm, C – A ≥ 5 mm, B – A, and D – C ≤ 4 mm → AmpC activity only

B–A ≥ 5 mm, D – C ≥ 5 mm, B – D, and C – A ≤ 4 mm → ESBL activity only.

D–C ≥ 5 mm, B – A ≤ 4 mm → ESBL, and AmpC combined activity.

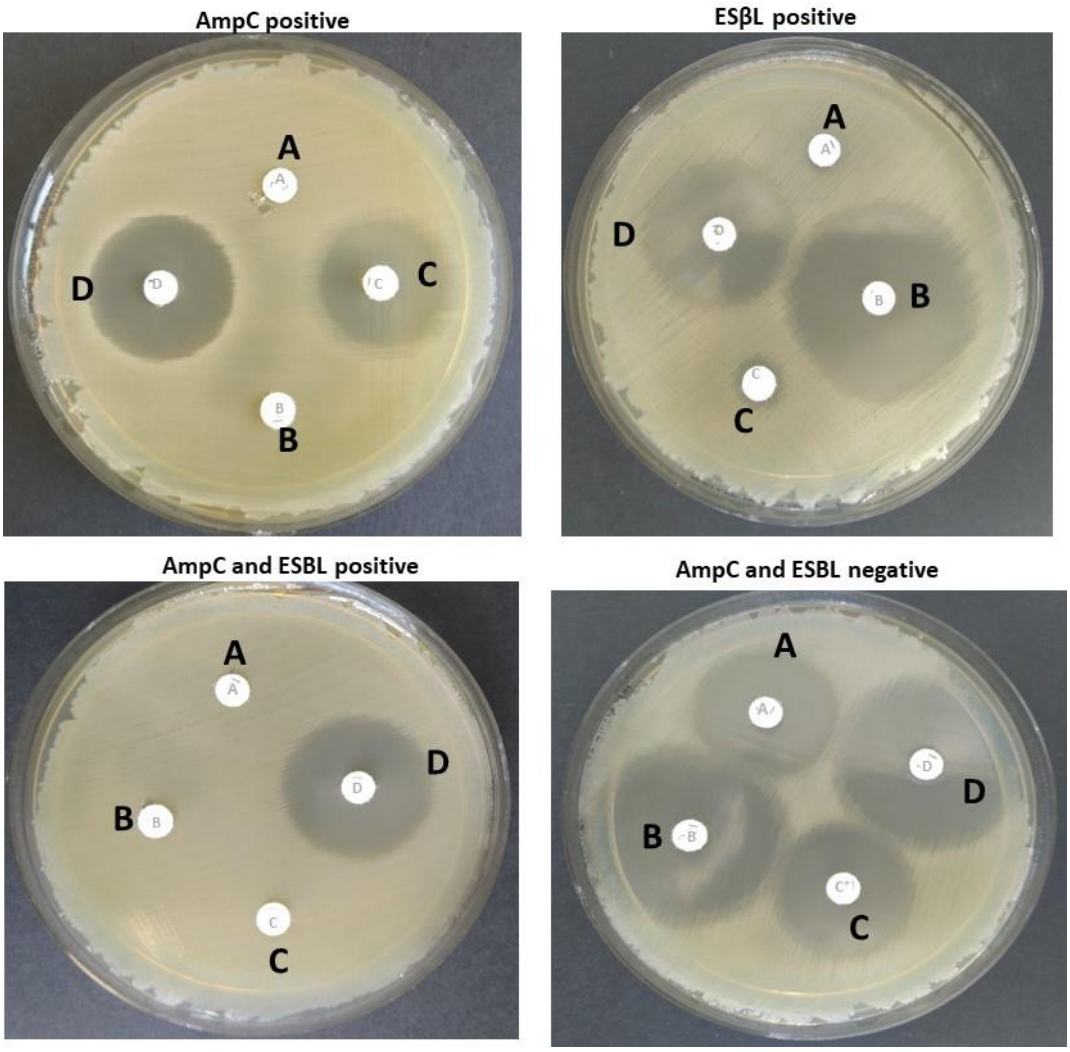


Figure 5.1. Phenotype screening for AmpC and ESBL producing dairy farm soil *E. coli* isolates

For the detection of AmpC with porin loss KPC and MBL activity, the D73C (MAST™ Group Ltd, Liverpool, UK) the interpretations were as follows:

B*–A* ≥ 5 mm; C*-A* and D*-A* < 5 mm → MBL activity.

C*–A*, ≥ 5 mm; B*-A, D*-A*< 5 mm → KPC activity.

Distances between discs A*, B* C*, and D* ≤ 2 mm and E < 10 mm → OXA-48 positive

Distances between discs A*, B*, C*, and D* ≤ 2 mm and E > 10 mm → AmpC, KPC, OXA-48 negative (Figure 5.2).

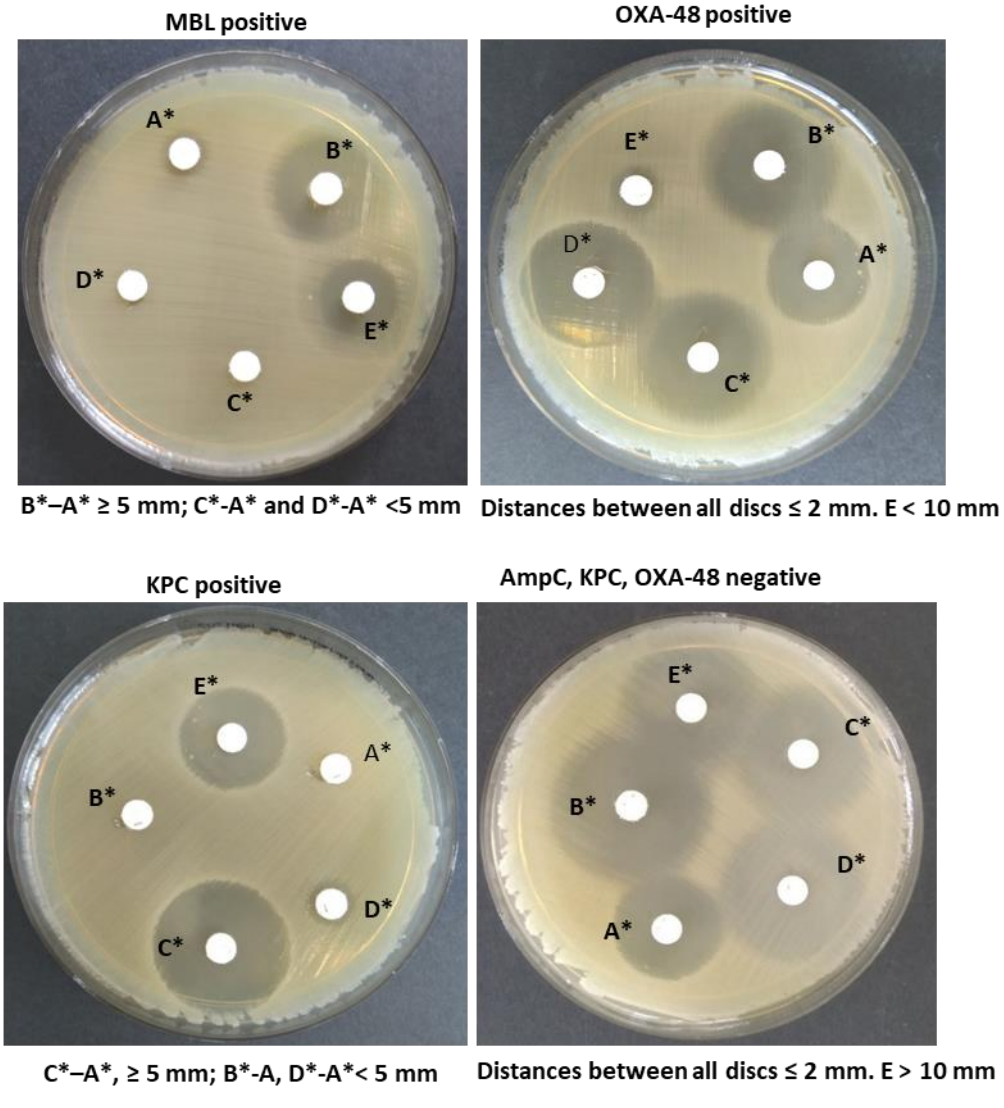


Figure 5.2. Phenotype screening for carbapenemase-producing dairy farm soil *E. coli* isolates

5.3 Results

The use of the CDDST D68C and the D73C (MAST™ Group Ltd, Liverpool, UK) kits enabled the confirmation of potential ESBL and AmpC β-lactamase producing DfSEC in this study. In this study, 31 isolates were phenotypically positive for the potential to excrete ESBL or AmpC β-lactamase or a combination of the two (Table 5.3). Out of the 31 isolates, 20 could additionally produce AmpC enzymes. Indicating the organism's ability to produce both AmpC and ESBL enzymes, similar to finding by Poulou et al. (2014) in a comparative study. The isolate TL56W18 was the only isolate that showed the potential to produce ESBL, KPC and AmpC hydrolysing enzymes, while PF55W18 was the only isolate to show the potential to produce ESBL, AmpC, and MBL (Table 5.3). It is worthy to note that, KPC, OXA, and MBL are all carbapenem hydrolysing β-lactamases (Naas, Nordmann, Vedel, & Poyart, 2005).

Statistical analysis by binary logistic regression in Minitab19 indicated that there were no significant differences ($P > 0.05$) between the two farming systems with regards to the number of isolates positive for the excretion of β-lactam hydrolysing enzymes only. However, an odds ratio of 1.5 indicated more isolates from the conventional dairy farms released β-lactamase compared to isolates from the organic dairy farms.

The only isolate out of 31 that was positive for the release of the OXA-48 hydrolysing enzyme, PF55W18 was from the Peel Forest conventional dairy farm with the zone diameter of > 10 mm to temocillin+MBL inhibitor. The OXA type β-lactamases are poorly inhibited by clavulanic acid (Naas & Nordmann, 1999) unlike ESBLs that are sensitive to clavulanic acid as an inhibitor (Poulou et al., 2014). Comparison between similar farming systems but different locations showed that the western located organic dairy farm of Totara Valley organic farm had eight isolates releasing ESBL, AmpC, or MBL hydrolysing enzymes with one isolate releasing resistant enzymes to multiple classes of antimicrobials while three isolates from the eastern located Clearwaters organic farm excreted only ESBL hydrolysing enzymes. In this study all isolates that showed phenotype characteristics of

resistance to the third generation cephamycin, FOX30 (Table 5.3) had their genome sequenced for molecular analysis (Chapter 7). According to Onishi, Daoust, Zimmerman, Hendlin, and Stapley (1974) cephamycins such as cephamycins B and C are 50 to 170 times respectively, more rapidly hydrolysed by β-lactamase produced by certain members of the Enterobacteriaceae family such as *Enterobacter. cloacae* compared to the cephamycin cefoxitin due to cefoxitin being a poor substrate for the β-lactamase these organisms produce.

Table 5.3. Phenotype screening for DfSEC and their resistance to cefoxitin 30 µg/disc (FOX30).

Using D68C and D73C* (MAST™ Group Ltd, Liverpool, UK)

		Differences in inhibition zones to D68C mm					Differences in inhibition zones to D73C* mm				FOX (30 mg) resistant	Resistant type
Farm	**Isolate**	**B-A**	**D-C**	**D-B**	**C-A**	**D-C**	**B*-A***	**C*-A***	**D*-A***	**E**	**≤ 10 mm**	**bla*ESBL***
Clearwaters (CW)	28-A18	7	0	1	8	0	2	6	6	21	+	ESBL+AmpC[#]
	33-S18	5	2	2	5	2	0	4	5	20	+	ESBL+AmpC[#]
	49-A18	3	5	6	4	5	0	6	5	22	+	ESBL+AmpC[#]
Mill Road (MRD)	21-A18	14	4	4	14	4	4	15	17	22	+	ESBL+AmpC[#]
	22-W18	6	0	0	6	0	2	2	2	17	+	ESBL+AmpC[#]
	24-S17	4	5	7	4	5	1	1	3	21	+	ESBL+AmpC[#]
	30-S17	6	0	1	7	0	3	2	2	21	-	ESBL
	33-S17	5	0	1	6	0	0	0	1	19	-	ESBL
	37-S17	5	0	0	5	0	0	1	1	25	-	ESBL

Table 5.3 continued

		Differences in inhibition zones to D68C mm					Differences in inhibition zones to D73C* mm				FOX (30 mg) resistant	Resistant type
Farm	**Isolate**	**B-A**	**D-C**	**D-B**	**C-A**	**D-C**	**B*-A***	**C*-A***	**D*-A***	**E**	**≤ 10 mm**	***bla*ESBL**
Peel Forest (PF)	17-S17	5	0	0	5	0	2	2	1	21	-	ESBL
	22-A18	5	2	3	6	2	0	0	0	25	-	ESBL
	24-A18	3	6	6	3	6	2	1	2	25	-	ESBL
	25-S18	6	5	4	5	5	1	4	2	20	+	ESBL+AmpC#
	32-A18	5	6	5	4	6	3	2	1	21	+	ESBL+AmpC#
	40-S17	5	0	1	6	0	0	1	0	22	-	ESBL
	45-S17	6	0	1	7	0	1	2	4	22	-	ESBL
	45-W18	3	1	1	3	1	4	6	4	24	+	ESBL+AmpC#
	52-W18	6	0	1	7	0	5	4	4	25	+	ESBL+AmpC#
	55-W18	7	0	1	8	0	2	2	2	8	+	ESBL+AmpC#+OXA-48
	30-A18	3	2	5	6	2	0	2	2	21	+	ESBL
	14-A18	7	0	1	8	0	4	11	4	22	+	ESBL+AmpC#
	15-A18	3	5	5	3	5	1	1	5	16	+	ESBL+AmpC#

Table 5.3 continued

		Differences in inhibition zones to D68C mm					Differences in inhibition zones to D73C* mm				FOX (30 mg) resistant	Resistant type
Farm	**Isolate**	**B-A**	**D-C**	**D-B**	**C-A**	**D-C**	**B*-A***	**C*-A***	**D*-A***	**E**	**≤ 10 mm**	***bla*ESBL**
Totara Valley (TL)	12-A18	1	6	6	1	6	1	1	0	22	+	ESBL+AmpC[#]
	1-S18	4	6	6	6	6	3	10	10	21	+	ESBL+AmpC[#]
	23-A18	4	1	5	6	1	2	2	1	21	-	AmpC
	2-A18	4	5	5	1	5	0	2	2	24	+	ESBL+AmpC[#]
	33-S18	5	0	1	6	0	4	2	4	20	-	ESBL
	54-S18	7	0	0	7	0	0	0	1	18	+	ESBL+AmpC[#]
	56-A18	6	2	0	4	2	12	3	4	19	+	ESBL+KPC+AmpC[#]
	87-A18	14	4	4	15	4	4	15	18	22	+	ESBL+AmpC[#]
	11-A18	7	1	1	7	1	1	1	1	20	-	ESBL

5.4 Discussion

In this study, a comparison between the inhibition zones around the disc with antimicrobial only and the synergistic disc with antimicrobial and the antimicrobial inhibitor helped to identify bacteria that release β-lactam hydrolysing chemicals as a mechanism of resistance. This procedure, however, does not indicate resistance due to the efflux pump, cell membrane impermeability, or change in the molecular structure of the β-lactam antimicrobial (Willems, Verhaegen, Magerman, Nys, & Cartuyvels, 2013). In this study, 20 DfSEC were resistant against FOX30 with inhibition zones ≤ 10 mm. Earlier studies used the inhibition zone of 18 mm (Onishi et al., 1974) and 4 µg/mL minimum inhibitory concentration (MIC) (Lepeule et al., 2012) as criteria for selection of resistance against antimicrobials of the cephalosporin group and cefoxitin of the cephamycins. In a comparative study of assays for the determination of AmpC β-lactamase producing *E. coli,* FOX(30) was found to be the discriminative parameter as 20 out of 21 (Peter-Getzlaff et al., 2011) and 55 out of 59 *E. coli* (Ghosh & Mukherjee, 2016) strains which were AmpC β-lactamase producers were resistant against cefoxitin. In another study on *E. coli* isolates from humans, cattle, and swine the production of AmpC β-lactamase was based on the resistance of the isolates to cefoxitin ≥8 µg/mL by the microdilution method (Johnson, Kuskowski, Owens, Gajewski, & Winokur, 2003).

E. coli may release hydrolysing enzymes including extended-spectrum β-lactamases such as KPC-lactamases, MB-lactamases, and OXA-48-lactamases to nullify the effects of not only β-lactam class of antimicrobials but other classes as well (Nordmann et al., 2012a; Nordmann, Dortet, & Poirel, 2012b; Nordmann, Naas, & Poirel, 2011), as was noted in this study. Otherwise, *E. coli* have the intrinsic ability to remove noxious substances by the efflux pump mechanism (Cox & Wright, 2013; Miller & Sulavik, 1996), reduce antimicrobial permeability into the cell (Knopp & Andersson, 2015; Lou et al., 2011) or modify antimicrobial target sites (Santajit & Indrawattana, 2016).

The phenotype screening results in this study compared to the original AST results may be explained based on;

a) AST does not involve the use of an anti-β-lactamase substance such as clavulanic acid, EDTA, or a boronic acid (Lalitha, 2004).

b) The various DfSEC may have possessed alternate resistant mechanisms, or under-expressed the antimicrobial-hydrolysing compound (Coyne, Guigon, Courvalin, & Périchon, 2010; Stuart & Leverstein-Van Hall, 2010; Yelin & Kishony, 2018).

c) Bacteria antimicrobial mechanisms may be over-expressed or under-expressed (Iredell, Brown, & Tagg, 2016; Yelin & Kishony, 2018) under different circumstances and conditions.

Besides, changes in environmental conditions such as growth rate, increased respiration, the formation of oxygen radicals, biofilm formation, and indirect resistance may affect the detection of a phenotype expression of resistance to an antimicrobial (Hughes & Andersson, 2017). Sánchez-Romero and Casadesús (2014) reported a heterogeneous expression of efflux pump activity among *Salmonella enterica* cells and colonies to nalidixic acid and suggested that adaptive resistance/susceptibility may explain differences in the phenotypic expression of resistance/susceptibility of certain bacteria to a particular antimicrobial at different times.

The CDDST or the DDST indicates the presence of the hydrolysing enzyme, but the release of these enzymes by the bacteria may be intermittently, or under-expressed in some cases (Pitout, 2012a; Sundin, 2009). This is similar to the sporadic expression of plasmid-mediated AmpC and other ESBL expressions in *E. coli* (Naseer, Haldorsen, Simonsen, & Sundsfjord, 2010). Further, the demonstration of AmpC resistance may be by outer membrane porin loss (Ananthan & Subha, 2005) and confer resistance to amino-penicillins, oxyamino-cephalosporins, cephamycins, and monobactams (Bush & Bradford, 2016; Maleki et al., 2015; Peter-Getzlaff et al., 2011). However, in a human UTI study, all AmpC producing *E. coli* were found to be ESBL producers (Gupta, Rani, Singla, Kaistha, & Chander, 2013). ESBLs cause resistance to all penicillins, third-generation cephalosporins such as ceftazidime, cefotaxime, and ceftriaxone, and aztreonam, but not to the closely related cephamycins such as cefoxitin (Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015). In this study, all DFSEC with ≥ 10 mm

inhibition zone diameter to FOX (30) were subjected to whole-genome sequencing (WGS) for molecular analysis to tease out the possibility of genotype-phenotype resistance(s) (Chapter 7).

Chapter 6

Evaluation of interrelatedness of dairy farm *E. coli* isolates using phylogenetic grouping

6.1 Introduction

Escherichia coli strains from various niches and sources have wide importance in both human and veterinary medicine, environmental sciences, genetics, and molecular biology application in research The characteristics and properties of *E. coli* whether as commensals or pathogens may be shared (Tóth et al., 2012) and transmitted along clonal lineages which can be identified as phylogenetic groups based on PCR methods (Agarwal et al., 2012; Alegría et al., 2020; Gordon, 2010; Tenaillon et al., 2010; Zhu Ge et al., 2014). For instance, the phylogenetic groups of *E. coli* (A, B1, B2, and D) (Gordon, Clermont, Tolley, & Denamur, 2008) and E and F (Clermont et al., 2013) are known to exhibit different phenotypic characteristics such as the difference in temperature/growth rate relationships, sugar metabolism, and AMR profiles (Gordon et al., 2008; Walk et al., 2007).

Escherichia coli strains may be grouped according to their serotype (Laing et al., 2012; Russo & Johnson, 2000), metabolic profile (Liao, Kim, & Tomb, 2002; Sousa, Hordijk, Steel, & Martin, 2015), and DNA hybridisation (Boileau, d'Hauteville, & Sansonetti, 1984; Guo et al., 2016; Moseley et al., 1980). Apart from these profiling methods, *E. coli* phylogenetic profiling has also brought more understanding to their evolutionary relatedness, especially, the pathogenic ones like the *Shigella* genus (Chaudhuri & Henderson, 2012) and enteroinvasive *E. coli* (EIEC) (Van den Beld & Reubsaet, 2012) using the molecular tool of wgMLST (Bai et al., 2019; van Hoek et al., 2019). The pulsed-field gel electrophoresis method is also widely used (Akindolire & Ateba, 2018; Ha et al., 2020). However, for organisms that tend to have multiple characteristics within a limited spread of strain variation, wgMLST is deemed more informative and the preferred technique (Harbottle, White, McDermott, Walker, & Zhao, 2006; Neoh, Tan, Sapri, & Tan, 2019) for interrelatedness and classification studies.

Apart from the immune status and other health aspects of an organism, the pathogenesis of an *E. coli* infection is based on the virulence of the strain (Phillips-Houlbracq et al., 2018; Riley, 2014). The association between a phylogenetic group and the severity and/or persistence of a disease like bovine mastitis cannot be ignored (Tomazi, Coura, Gonçalves, Heinemann, & Santos, 2018). For instance, bovine mastitis studies have found the *E. coli* phylogenetic groups of A and B1 to be most commonly associated with the disease (NandaKafle et al., 2017; Sheldon et al., 2010b; Suojala et al., 2011; Valat et al., 2012). Similarly, according to Liu et al. (2014), *E. coli* strains of the phylogenetic group A tend to harbour the highest number of virulence genes and cause the majority of acute bovine mastitis compared to the other groups. The association between *E. coli* phylogenetic groups and pathogenicity can be found in other organisms as well. For instance, in humans, and poultry the phylogenetic groups B2 and A, respectively are more pathogenic compared to the other groups (Jakobsen et al., 2010; Micenková et al., 2016).

Clermont, Bonacorsi, & Bingen (2000) proposed the use of the *chu*A, *yja*A genes, and TSPE4.C2 DNA fragment in a triplex PCR process in assigning 230 *E. coli* strains which had previously been grouped into their various phylogenetic groups, in a validation experiment. These *E. coli* strains were put into these phylogenetic groups based on the presence/or absence of the *chu*A, *yja*A, and TSPE4.C2 genes. Various authors have used this method of *E. coli* phylogenetic determination to align pathogenomic determinants of *E. coli* in their studies: Valat et al. (2012) used this triplex PCR protocol to assign 204 ESBL producing *E. coli* strains from cows with diarrhoea to their phylogenetic groups of A, B1, B2, D, and the virulent factor (VF) encoding genes. The authors were able to determine the percentages of the 204 strains that carried the *bla*CTX-M-15 IncI1-type plasmid. Australian researchers, Obeng, Rickard, Ndi, Sexton, & Barton (2012) used this method to place 251 *E. coli* strains from a poultry source into phylogenetic groups while assessing the virulence factors and antimicrobial-resistant profile of the *E. coli* strains to some selected antimicrobials including ciprofloxacin, florfenicol, gentamicin, streptomycin, and tetracycline. The link between ExPEC (extra-intestinal pathogenic *E. coli*) from humans with animal origin was commented on by analysing the similarity of ExPEC strains

by molecular method. The Clermont et al. (2000) triplex PCR phylogenetic analysis was used by Bélanger et al. (2011) in their study.

Gordon et al. (2008), evaluated the accuracy of the Clermont et al. (2000), triplex PCR method by comparison of the *E. coli* multilocus sequence typing (The French MLST http://www.pasteur.fr/mlst and The German http://web.mpiib-berlin.mpg.de/mlst/dbs/Ecoli schemes) with the triplex PCR phylogenetic typing of 662 *E. coli* strains from varying sources. Their results indicated a 95% consistency between the two methods (Gordon et al., 2008). It was also found that 9% of the strains that were assigned phylogenetic group A were incorrectly grouped. Secondly, some strains that were originally assigned to phylogenetic group A based on the null presence of the three genes (*chu*A-, *yja*A- and TSPE4.C2) should, in reality, have been classified as 'non-assignable'. Also, some strains that were triplex PCR assigned to phylogenetic group D were unassigned or put into a new phylogenetic group, E (Gordon et al., 2008), citing (Escobar-Páramo, Clermont, & Blanc-Potard, 2004) and (Selander, Caugant, & Whittam, 1987)

Despite its extensive use, a search through the GenBank databases by Doumith, Day, Hope, Wain, & Woodford (2012) revealed some nucleotide polymorphism within the annealing regions of the PCR primers for the three genes used for the Clermont et al. (2000) triplex PCR. This, according to Doumith et al. (2012), causes some *E. coli* strains to be erroneously assigned to phylogenetic groups. The use of an internal control primer of the *E. coli* glutamate decarboxylase (*gad*) gene as well as using newly designed primers was proposed (Doumith et al., 2012). Improvement in the accuracy of assigning strains to their correct phylogenetic groups was noted when the Clermont et al. (2000) method was compared to the new quadruplex PCR method and compared by GenBank BLAST (Doumith et al., 2012). Overdevest et al. (2015) used the Doumith et al (2012) quadruplex PCR protocol to accurately find the association between the phylogenetic grouping of 108 wild-type *E. coli* and 134 ESBL producing *E. coli* strains in a study. Similarly, Freitag, Michael, Kadlec, Hassel, & Schwarz (2017) used the same quadruplex PCR method in a study on 878 *E. coli* isolates from cows with mastitis to accurately assign the isolates to their phylogenetic groups.

Following the discovery of the shortfalls in the Clermont et al. (2000) triplex PCR method a review was carried out by Clermont et al (2011). The original *chu*A, *yja*A, and TspE4.C2 genes were maintained and changes were made to the primer sequences to avoid polymorphism during the annealing stage of the PCR. The phylogeny groups of clade I, III, IV, and V were also classified (Clermont et al., 2013). Apart from these changes, a new *arp*A gene was included with the previous three genes to develop a new quadruplex PCR (Clermont et al., 2013). The inclusion of the *arp*A gene was used as an internal control and as a tool to distinguish between strains which would otherwise be classified as phylogenetic group D or B2 into a new phylogenetic group, F. Further, when an absence of the *arp*A gene in the new quadruplex PCR product was noted, it helped to put strains into cladesII, III, IV, and V, otherwise, the organisms were identified as either *Escherichia albertii* or *Escherichia fergusonii*.

After validating the quadruplex PCR method, Clermont et al. (2013) proposed a new *E. coli* phylogenetic grouping of A, B1, B2, C, D, E, F, and clade I-V. This method and proposal have since been widely accepted and used by various researchers around the world (Beattie et al., 2020; da Silva, de Mello Santos, & Silva, 2017; Hassen et al., 2020; Iranpour et al., 2015; Kumar, Nahid, & Zahra, 2017; Kuznetsova et al., 2018; Massot et al., 2016; Müştak et al., 2015; Rana et al., 2017; Tomazi et al., 2018) and improved upon with a web-based computerised approach (Beghain, Bridier-Nahmias, Le Nagard, Denamur, & Clermont, 2018).

In this chapter, the phylogenetic relationship among *E. coli* strains recovered and characterised from each of the four farms examined (as described in Chapters 2-5) were examined, to evaluate any possible interrelationship between them (given the proximity of the sites), resistance traits and/or human cases, using the validated Clermont et al. (2013) method and subsequently wgMLST, for their interrelatedness (Chapter 7).

6.2 Materials and methods

6.2.1 Isolates

In this study 814 dairy farm *E. coli* (DfSEC) isolates were grouped into groups A, B1, B2, C, D, E, or clade I, II, III, IV, or V phylogenetic of *E. coli* using primers and the protocol described by Clermont et al. (2013).

6.2.2 PCR phylogenetic typing

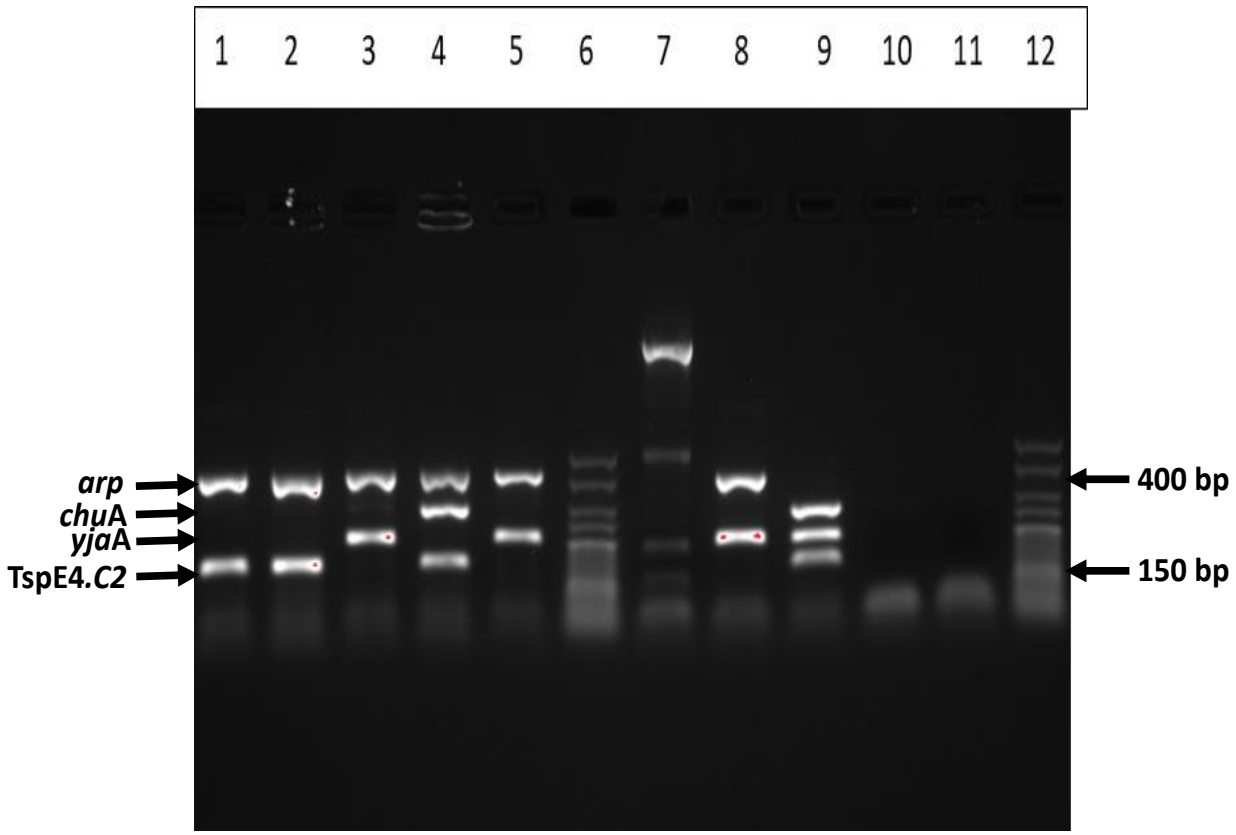
Escherichia coli cells from a 24 h old single colony from a TBX agar plate were suspended in PBS at a concentration equivalent to 0.5 McFarland standard. The cell suspension was heat-lysed (Brian et al., 1992) at 95°C for 5 min in a heat block (AcuBlock™ Labnet International INC NJ, USA) and centrifuged (Eppendorf® Minispin® plus, Sigma-Aldrich, Auckland, NZ) at 12 000 g for 5 min. The supernatant was used as the source of DNA for the PCR reaction. A 20 µL PCR reaction was set up using 2 µL of 10X PCR buffer, 0.4 µL of 2 µM each of dNTP, 0.4 µL of Taq polymerase, 2 µL each of primer's forward (f) and reverse (r) for *chu*A1 (f), *chu*2 (r), *yj*A, *Trp* at 20 pmol each. For *Acek* (f) and *Arp*(r) at 20 pmol each, however, 4 µL of primers were used per reaction. The reaction mixture was completed with 1.2 µL of 25 mM $MgCL_2$, 2 µL of molecular grade distilled water, and 1 µL of the DNA template (Clermont et al., 2013; Lescat et al., 2013).

The reactions were set in a thermocycler (Labnet MultiGene TC 9600G Sigma-Aldrich, Auckland, NZ) for 30 cycles of denaturing 94°C for 5 min followed by annealing at either 57°C for 2 s (for group D+E) or 59°C for 2 s for quadruplex and A+C differentiation and extension at 72°C for 5 min. The final product was stored at 4°C until the PCR product was run on 2% agarose gel (2 g molecular grade agarose to 100 mL of 1M tris EDTA buffer) electrophoresis using 1X TBE buffer and 0.07 µL Sybrsafe (Invitrogen, Auckland, NZ)/mL of gel, run at 90 V for 60 min, visualised and photographed with a molecular imager (Gel Doc™ XR+ Bio-Rad Laboratories Pty. Ltd, Auckland, NZ). Statistical analysis was done using t-tests or ANOVA, as appropriate, in SigmaPlot14.0 statistical software.

6.2.3 Phylogenetic group assignation

In this study, the phylogenetic group assignation was done according to the guidelines of Clermont et al. (2013). Briefly, the presence (+) or absence (-) of a quadruplex PCR product band in a lane corresponding to an isolate was marked according to the molecular mass of the band. The phylogenetic group was assigned according to the final analyses of the band's absence/presence (-/+) as outlined by Clermont et al. (2013) (Figure 6.1 and Appendix C1), using the hyperladder™ V (HVL) (Bioline, Meridian® Biosciences, Total Lab Systems Ltd, Auckland, New Zealand). In cases where an isolate was initially typed by the quadruplex PCR as either the A+C phylogenetic group or the D+E phylogenetic group, a second PCR was run using the primers *trpAgp*C.f, *trpAgp*C.f and *ArpAgpE*.f, *ArpAgp*.r, respectively. The primers *trp*BA.f and *trp*BA.r were included as an internal control which also helped to differentiate between phylogenetic group E and clade I+II (Clermont et al., 2013).

An isolate, when assigned a preliminary group of D+E, E+Clade I+II, or A+C was re-assigned its final phylogenetic group after confirmation or denial of the presence of a band when a secondary PCR with the trp*Agp*C or try*Agp*E primer in a duplex PCR reaction corresponding to a group C or E, respectively instead of a group A or D accordingly. Based on the reviewed *E. coli* phylogenetic group method, Clermont et al. (2013) constructed a minimum spanning tree to show the phylogenetic groups of a subset of *E. coli* strains originally misclassified by the triplex PCR method (Figure 6.2). Statistical analysis of the phylogenetically typed 814 *E. coli* isolates collected from the four farms over the four-time points was done using the Mann-Whitney Rank Sum t-test or by Kurskal-Wallis ANOVA on ranks, as appropriate, in SigmaPlot14.0.



arp	+	+	+	+	+		-	+	-	-	-	
***chu*A**	-	-	-	+	-		-	-	+	-	-	
***yja*A**	-	-	+	-	+		-	+	+	-	-	
tspE4.C2	+	+	-	+	-		-	-	+	-	-	
Group	B1	B1	A+C	D+E	A+C	HVL	U	A+C	B2	nil	nil	HVL

U=unknown
HVL=hyperladder™V

Figure 6.1 *E. coli* quadruplex phylogenetic group assignation

Based on (Clermont et al., 2013)

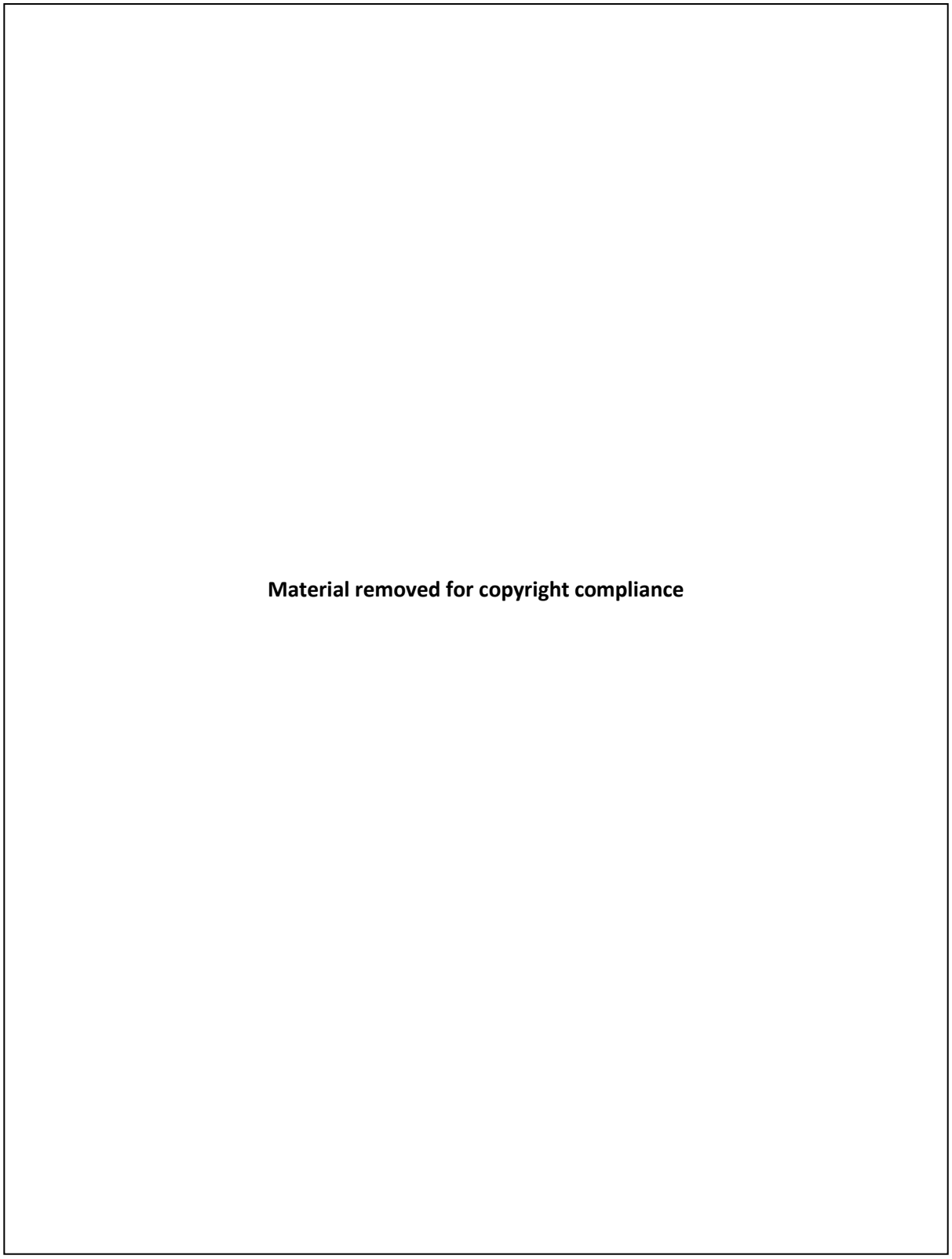


Figure 6.2 Phylogenetic group tree of *E. coli* isolates after modification of modified Clermont et al. (2000)

Isolates in red were originally, erroneously assigned to group D using the Clermont et al. (2000) method. According to Clermont et al. (2013)

6.3 Results

The phylogenetic grouping, using the Clermont et al. (2013) protocol for 814 DfSEC isolates isolated from the dairy farm soil in 2017-2018 (Chapter 2) is shown in Table 6.1. The B1 phylogenetic group predominated at 73.7% of the 814 dairy farm soil isolates. The E phylogenetic group at 9.6% was the next most common, followed by group phylogenetic A at 5.8% and group C at 5.3%. The clade I+II and cladeIII+IV+V groups were 0.7% and 0.9% of the total, respectively. The B2 and D groups each represented 0.5% of the 814 isolates. None of the DfSEC were of the F group and 3.1% of the isolates could not be placed in any of the presently recognised *E. coli* phylogenetic groups using the Clermont et al. (2013) protocol.

Statistical analysis showed no significant difference between the phylogenetic groups of the two farming systems of conventional and organic dairy farming. Also, there were no significant differences in the phylogenetic groupings between the farms. Similarly, there were also no significant differences between the phylogenetic groups according to the four sampling times by the Kurskal-Wallis ANOVA on ranks SigmaPlot 14.0 statistical analysis.

In this study, the aim of the phylogenetic grouping of the DfSEC isolates was to evaluate the phylogenetic relationships among the 814 DfSEC isolates obtained to ascertain any possible linkages between isolates from differing dairy farming of the AMR *E coli* isolates. Also, the phylogenetic grouping could help elucidate possible links between the DfSEC and other mammals including humans and their companion animals that may have interaction with the cows in their environment on a typical dairy farm in the Geraldine district and New Zealand in general. Thirdly, to assess the possibilities of phylogenetic grouping with causative agents of common dairy farming diseases such as mastitis and metritis.

Table 6.1 The phylogenetic groupings of 814 confirmed dairy farm soil *E. coli* isolates collected over 4-seasons.

		Phylogenetic group									
Farm-season-year	(n)	A	B1	B2	C	D	E	F	Clade I,II	Clade III-V	Unknown
CW-S-17	50	2	45	0	1	0	2	0	0	0	0
CW-A-18	50	1	40	0	6	0	2	0	0	0	1
CW-S-18	47	0	28	0	5	1	13	0	0	0	0
CW-W-18	47	4	26	0	2	1	13	0	0	1	0
TL-S-17	51	0	34	0	5	0	4	0	0	0	8
TL-A-18	47	3	36	0	0	0	0	0	1	0	7
TL-S-18	50	1	46	0	0	0	24	0	0	1	0
TL-W-18	47	7	35	0	0	0	5	0	1	1	3
MRD-S-17	68	4	54	2	3	0	4	0	0	0	1
MRD-A-18	55	4	42	1	0	1	6	0	1	1	0
MRD-S-18	50	2	38	1	3	0	6	0	0	0	2
MRD-W-18	50	9	32	0	0	0	9	0	0	0	3
PF-S-17	50	1	29	0	13	0	6	0	1	0	0
PF-A-18	55	0	38	0	3	1	9	0	2	2	0
PF-S-18	49	3	38	0	1	0	6	0	0	1	0
PF-W-18	48	6	39	0	1	0	2	0	0	0	0
Total	814	47	600	4	43	4	78	0	6	7	25
%		**5.8**	**73.7**	**0.5**	**5.3**	**0.5**	**9.6**	**0.0**	**0.7**	**0.9**	**3.1**

CW=Clearwaters organic dairy farm: TL=Totara valley organic dairy farm: MRD=Mill road conventional dairy farm: PF=Peel Forest conventional dairy farm: S-17=spring of 2017: S-18=spring of 2018: A-18=autumn 2018: W-18=winter of 2018

6.4 Discussion

In this study, the *E. coli* phylogeny group B1 was the predominant group in the 814 DfSE isolates collected from the dairy farms. This was similar to other studies that have looked at the phylogenetic grouping of *E. coli* isolates of bovine origin (Blum & Leitner, 2013; Milanov, Prunic, Velhner, Todorovic, & Polacek, 2015; Suojala et al., 2011). The phenomenon of different members of the various *E. coli* phylogenetic groups dominating in prevalence among a particular species of animals and humans as well as niches has been demonstrated in numerous studies (Ewers, Antão, Diehl, Philipp, & Wieler, 2009; Ewers, Bethe, Semmler, Guenther, & Wieler, 2012). Even for a particular animal species, the distribution of the various phylogenetic groups as commensals or pathogens may belong to different phylogenetic groups (Jang et al., 2017; Keane, 2016; Mercat et al., 2016; Suojala et al., 2011). This is true for the *E. coli* associated with cattle. Studies that dealt with the *E. coli* from the bovine environment such as soils and manure have indicated the phylogeny group B1 to be most predominant (Blum & Leitner, 2013; van Overbeek et al., 2020). This is similar to results in this study with the B1 group dominating at 73% of the DfSEC isolates.

Mastitis is the most concerning pathology on a dairy farm (Ruegg, 2017; Ruegg & Petersson-Wolfe, 2018; Ruegg & Reinemann, 2002). In New Zealand, about 14/100 cows/annum of the milking herd on a bovine dairy farm would be affected by mastitis (McDougall & Compton, 2002). The best treatment of mastitis is the use of antimicrobials because the main causative agents are bacteria including *E. coli* (McDougall, 2002). In most OECD countries including New Zealand, milk from cows with mastitis being treated with antimicrobials must be disposed-off, until the withholding period of the drug is over (Anika et al., 2019). The milk is either fed to calves on the farm or disposed of in the sewage (Lago, Godden, Bey, Ruegg, & Leslie, 2011; Ruegg, 2017; Ruegg & Reinemann, 2002). The mastitis-causing bacteria from such milk may thus end up in the soils of the fields, through the digestive system of the calves and the sewage used for irrigation (Houlbrooke, Horne, Hedley, Hanly, & Snow, 2004). Polacek (2015) explained that pathogenic *E. coli* strains possess special features like *curli* fimbriae for adhesion, invasion of host cells and to protect themselves with biofilm formation to enable them to persist in the mammalian system to avoid destruction by antimicrobials. Such soil *E.*

coli isolates may easily penetrate mammalian cells with such features to cause infection and be able to avoid destruction by antimicrobials (Milanov et al., 2015).

In this study, the second most common of the *E. coli* phylogeny groups was type E at 5.9% using the Clermont et al. (2013) method. This method can tease out *E. coli* isolates that were previously grouped into group D+E into either D, E, or clade II+III+IV and A+C into either A or C groups as opposed to a previous phylogeny typing method that other authors have used (Gordon, 2010). Authors who have used the less sensitive Clermont et al. (2000) method have indicated the phylogeny group D to be next in common following groups B1 and A in pathological cases of mastitis (Suojala et al., 2011; Zhang et al., 2018) and metritis (Gonzalez Moreno, Torres Luque, Oliszewski, Rosa, & Otero, 2020; Silva et al., 2009). Similarly, while the phylogenetic group A had featured in most studies, group C had had little mention but, in this study, 5.3% of the DfSEC belonged to the C group. This may be because, in this study, DfSEC isolates that would have been typed as A or A+C were re-typed with the trp*Agp*C primers to differentiate A+C into As and Cs. Other studies had used the earlier version, Clermont et al. (2000), typing protocol and had not been able to differentiate some *E. coli* isolates into their phylogenetic groups as robustly as provided by Clermont et al. (2013). This was shown by Logue et al. (2017) in a study.

According to Blum and Leitner (2013) and Kempf, Slugocki, Blum, Leitner, and Germon (2016) the *E. coli* phylogenetic group A is most commonly associated with mastitis and metritis (inflammation of the endometrium), respectively. In this study, it may be argued that the members of the phylogeny group A, may have originated from the mammary glands of the cows with mastitis or from secretions and/or aborted foeti from cows with metritis because of the pathogenic traits they displayed, and the virulent determinant carried. Also, in this study, the phylogenetic group B2 was 0.5% of the 814 DfSEC isolates and may have originated from humans, cats, and/or dogs (Bogema et al., 2020; Collis et al., 2019; Kidsley et al., 2020; Toombs-Ruane et al., 2017). This is because, the phylogenetic group B2 is hardly associated with bovine (Liu et al., 2014; Madec et al., 2012) but it is the predominant group associated with humans and their companion animals of cats (Zogg, Zurfluh, Schmitt, Nüesch-

Inderbinen, & Stephan, 2018) and dogs (Harada et al., 2012; Mateus et al., 2013), common on New Zealand dairy farms.

In New Zealand, some *E. coli* and Shiga toxin-producing *E. coli* (STEC) O157:H7 and other bacterial infections in humans have been attributed to contact with farm animal subjects and/or their products with cattle as the principal source of these infections (Gilpin, Scholes, Robson, & Savill, 2008; Gilpin et al., 2020; Jaros et al., 2013). This study highlights such a possibility and also the possibility of humanly sourced infectious *E. coli* getting into the cattle environment of a dairy farm soil. This is explained by *E. coli* of phylogenetic A and B2 being most commonly associated with humans but rarely with bovine (Pietsch et al., 2018).

Chapter 7

Whole-genome sequencing of dairy farm soil *E. coli* isolates producing Extended Spectrum β-lactamases.

Whole-genome sequencing (WGS) offers the most sensitive and specific approach in determining the resistant gene(s) encoded by bacteria (Ellington et al., 2017). Pulsed-field gel electrophoresis (PFGE) has been used by many researchers over the years. It continues to be used to identify clones that may frequently be antibiotic-resistant, it lacks the sensitivity and specificity offered by WGS, especially if the organisms share ancestry (Ruppitsch et al., 2015; Salipante et al., 2015).

In research cases, where diversity, origin, and epidemiological data are of relevance, WGS offers the best approach even when compared to multi-locus sequence typing (Chen, Karanth, & Pradhan, 2020; Egan et al., 2020; Liu et al., 2020; Lytsy, Engstrand, Gustafsson, & Kaden, 2017; Walker et al., 2013). It is well noted, however, that obtaining, analysing, and implementing the information from a WGS is not trivial (Carriço, Rossi, Moran-Gilad, Van Domselaar, & Ramirez, 2018) despite computer software and interpretation guides (Kozyreva et al., 2017; Lindsey, Pouseele, Chen, Strockbine, & Carleton, 2016; Rouard et al., 2019; Wyres et al., 2014).

From 2001 to the present, there has been a huge reduction in the cost, decrease in turn-around time and the increase in quality of WGS outputs, as well as an increase in the number of WGS service providers and the availability of equipment and reagents both for the preparation of samples for WGS and the running of it (Gullapalli, Desai, Santana-Santos, Kant, & Becich, 2012; Koboldt, Steinberg, Larson, Wilson, & Mardis, 2013; Shang et al.). There are also commercially available kits for the extraction and preparation of high-quality genomic DNA samples for WGS at reasonable costs, easy-to-follow instructions, and have a long shelf life.

The human-genome project, which was completed in 2001 after 20 years of work and over 3 billion USD spent, elevated WGS into a new sphere of molecular science. One of the key by-products of the project was the reduction in the cost of WGS (Lander, 2011; Lander et al., 2001).

In this study, 20 DfSEC isolates that showed the potential to release ESBLs enzymes and had an inhibition zone to FOX30 of ≥ 10 mm after phenotype screening (Chapter 5) were subjected to WGS and whole genome multi-locus sequence typing (wgMLST) analysis to confirm the resistance genotype and investigate the presence of other genetic AMR traits.

7.1 Materials and methods

The genomic DNA template for WGS was extracted using the column-based genomic DNA extraction kit, GenElute™ Bacterial Genomic DNA kit Sigma-Aldrich, Auckland, NZ. Briefly, a single colony of 24 h old DfSEC (previously species identified by PCR and stored at -80°C, Chapter 2) from a TBX agar plate was streak-plated onto nutrient agar and incubated at 37°C for 24 h. A single colony from the nutrient agar plate was put into 1 mL of molecular grade pure water and washed by centrifugation of cell suspension at 12 000 g for 2 min. The use of TBX agar plate gave instant recognition of possible contaminants without the need for UV lamp as in NMUG usage, while the final use of nutrient agar prevented the blue colouration from TBX agar, possibly interfering with DNA concentration determination by a spectrophotometer (Sánchez-Fito & Oltra, 2015) such as a Nanodrop (DeNovix DS-11+ Spectrophotometer, dNature Diagnostics& Research Ltd, Gisborne, NZ).

The resulting pellet of the washed *E. coli* cells was re-suspended in 180 µL of the GenElute™ lysis solution. Then, 20 µL of RNase solution was added to the lysed cell solution to help remove RNA contaminants and incubated at room temperature for 2 min. A 20 µL aliquot of proteinase K solution was added to the sample, vortexed gently for 30 s and incubated at 55°C for 30 min. 200 µL of a second GenElute™ lysis solution was then added to the sample after the initial 30 min incubation at 55°C and incubated further at 55°C for 10 min. A homogenous mixture was obtained. Subsequently,

200 µL of absolute ethanol was added to the lysate, and the mixture vortexed for 8 s, a homogenous mixture resulted.

A GenElute™ column was prepared for DNA binding by adding 500 µL of GenElute™ column-preparation solution to the column and centrifuged at 12 000 g for 1 min. The flow-through was then discarded. The prepared binding column was mounted into a 2 mL Eppendorf tube as the collection tube and the cell lysate was put into the column. Using a 1 000 µL pipette tip with a wide enough tip to avoid shredding of the DNA product, the contents of the sample mixture were gently pipetted onto the prepared column in a 2 mL Eppendorf tube and centrifuged at > 6 500 g for 1 min. The flow-through was again discarded but the column was maintained. A 500 µL of GenElute™ solution1 and a wash solution were added onto the column and span at > 6 500 g for 1 min and the flow-through was discarded. Finally, 200 mL of a GenElute™ elution buffer was added to the column and span at 6 500 g for 1 min.

The resulting solution of genomic DNA was tested for quality, and purity-checked using a Nanodrop (DeNovix DS-11+ Spectrophotometer, dNature Diagnostics & Research Ltd, Gisborne, NZ). Then, 5 µL of the extracted genomic DNA was again checked for quality and purity on a 0.8% agarose gel using SybrSafe to aid visualisation and photography by an imager (Gel Doc™ XR+ Bio-Rad Laboratories Pty. Ltd, Auckland, NZ).

For the WGS, DNA libraries were prepared using Nextera® XT v2 Library Preparation Kit (Illumina, San Diego, USA) and pooled libraries were sequenced on a NextSeq 550 sequencer (Illumina). Sequences were quality checked and evaluated using Nullarbor. The genomes were assembled using SPAdes within BioNumerics 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). A range of phenotypes was predicted both within BioNumerics and through Nullarbor (Nullarbor Github: https://github.com/tseemann/nullarbor). Whole-genome Multi-Locus Sequence Typing (wgMLST) was performed in BioNumerics. The BioNumerics of *E. coli* wgMLST scheme indexes sequence differences for up to 17 380 genes. A wg-MLST minimum spanning tree was generated for 20 DfSEC isolates. In this study, these isolates were chosen based on their zone of inhibition to cefoxitin 30 mg

(FOX30) being as low as ≤ 10 mm after disc diffusion antimicrobial susceptibility testing according to the protocol outlined by EUCAST (2019). The genomes were exported from BioNumerics in Fasta format and submitted to the PubMLST rMLST species identification website (https://pubmlst.org/bigsdb?db=pubmlst_rmlst_seqdef_kiosk) as another form of identification. This aspect of the study was contracted out to ESR, Christchurch, NZ for processing.

The concatenated contiguous sequences of the 20 DfSEC isolates were loaded into Geneious™ Pro. The nucleotide sequence of the open reading frame (ORF) of ESBL genes evaluated by Dallenne, Da Costa, Decré, Favier, and Arlet (2010) (Table 7.1) were obtained from the NCBI website in Fasta format. The nucleotide sequence of each gene's ORF was queried against the sequenced assembly of each of the 20 DfSEC isolates for translated amino acid matches using the Geneious™ Pro bioinformatics software. This was similar to the ResFinder for determination of ARGs, with coverage identity set at 98% (but possible at 80%-100%) to help remove noises such as the fragment of genes (Zankari et al., 2012). In this study, significant hits defined as > 80% query coverage with > 70% pairwise identity (Kluytmans-van den Bergh et al., 2016) were then used as queries for the Basic Local Alignment Search Tool (BLAST) searches of NCBI protein conserved domain for protein (Lu et al., 2020) database(s) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to identify the protein (enzyme) they encoded. To confirm that the enzyme that resulted from the BLAST NCBI search query was of relevance as a genotype-phenotype determinant (Hendriksen et al., 2019), the nucleotide sequence of a particular gene's ORF was first BLAST queried. The resulting enzyme's accession number, name, and description were compared to the enzyme resulting from each DfSEC isolate's BLAST NCBI query.

Briefly

- The "Fasta" format of the nucleotide sequence of the ORF of a gene variant was queried for the protein (enzyme) that is encoded in the NCBI conserved domain site.
- The protein name, code, and accession number, as well as the E-value, were recorded
- The nucleotide sequence of the ORF of a gene variant was queried against the concatenated sequence of a DfSEC's nucleotide sequence (assembly).

- The contig number, coordinates, coverage, and pairwise percentages were noted.
- The amino acid of matching pair of > 80% coverage and > 70% pairwise identity of a gene variant's ORF versus concatenated nucleotide sequence of DfSEC query was copied from Geneious™ Pro result output (Zankari et al., 2012).
- The copied amino acid sequence in Fasta format was queried in the NCBI conserved domain similar to the procedure for the Fasta format of the ORF of the gene variant in question (Table 7.2-7.8).

The phylogenetic tree was created in Geneious™ Pro using the matching nucleotide sequence of the DfSEC isolates and that of the various AMR genes that had significant coverage and pairwise identity of > 83% and > 70% respectively. In Geneious™ Pro, the cost matrix of 65% (5.0/-0.4) with a gap penalty of 12 or 6, gap extension penalty of 3, the global alignment with free end gaps, using the Tamura-Nei genetic distance model to build a neighbor-joining tree with no out-groups. These parameters were similar to parameters set by Wylie, Luo, Li, and Jones (2012) in a study and Harris, Balcerzak, Johnston, Schneiderman, and Ouellet (2016) in another.

Table 7.1. Beta-lactamase genes used for Geneious™ Pro 5.6.7 analysis

***bla* gene**	**variant**	**accession number**
TEM	1	EF125012
	29	DQ269440
SHV	1	AF148850
	2	AY570959
OXA	1	JO2967
	48	AY236073
CTX-M	1	X92505
	3	Y10278
	15	AY044436
	2	AJ416345
	9	AJ416345
	14	AJ972957
	8	AF189721
	25	AF518567
	26	AY455830
	39	AY954516
	40	AY750914
	41	DQ023162
ACC	1	AJ133121
	2	AF180952

Table 7.1 continued

***bla* gene**	**variant**	**accession number**
FOX	1	X77455
	2	Y10282
	3	Y11068
	4	AY007369
	5	AY007369
MOX	1	D13304
	2	AJ276453
CMY	1	X92508
	2	DQ355981
	4	AJ00726
	8	AF167990
	9	AB061794
	10	AF381618
	11	AF381626
	19	AB194410
DHA	1	EF406115
	2	AF259520
BIL	1	X74512
ACT	1	U58495
MIR	1	M37839
VEB	1	AF324833
PER	1	Z21957

Table 7.1 continued

***bla* gene**	**variant**	**accession number**
GES	1	AF156486
	2	AF326355
IMP	1	S71932
	7	AF416736
	10	AB074433
VIM	1	AJ291609
	2	AF3055559
	4	EF467306
KPC	1	AF297554
	2	DQ989640

7.2 Results

7.2.1 DNA extraction

A clear band with no smearing was obtained for all 20 samples (Figure 7.1) when the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, Auckland, NZ) was used to extract the genomic DNA from the 20 selected samples for WGS. The concentration and ratio of all samples submitted for WGS was > 10 µg/mL and the ratio of 260/280 nm by a Nanodrop (DeNovix DS-11+ Spectrophotometer, dNature Diagnostics& Research Ltd, Gisborne, NZ) was between 1.80 and 2.00.

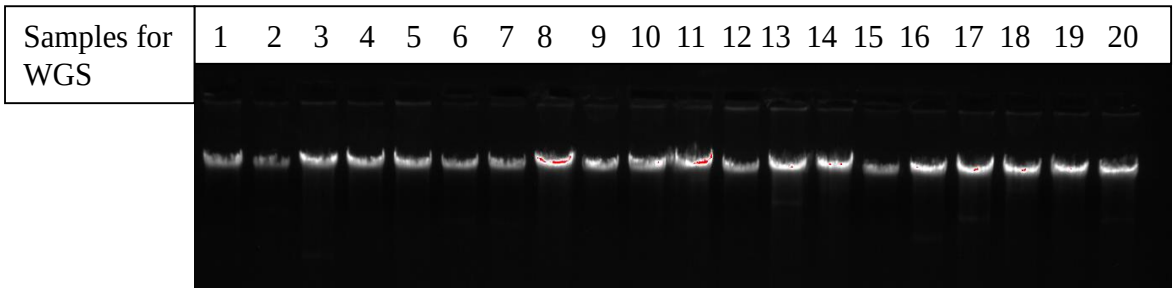


Figure 7.1 Gel electrophoresis showing DNA extracted from selected dairy farm soil *E. coli* isolates.

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7.2.2 Resistant genes analysis of DfSEC isolates

The 20 DfSEC isolates submitted for wgMLST encoded at least one of 12 different ARGs (Table 7.2) according to the Nullarbor bioinformatics software analysis. From the two organic dairy farms, all nine (100%) of the isolates studied encoded two or more resistance genes. The isolates CW33S18, TL1S18, and TL2A18 encoded three ARGs. Similarly, all DfSEC isolates encoded two or more ARGs according to the Nullarbor analysis. The isolates PF14A18 and PF15A18 encoded the most number of ARGs at 7/12 while the isolate PF55W18 encoded 6/17 resistant genes and PF32A18 encoded 4/12 ARGs. Out of the 20 DfSEC isolates 18 encoded the *mdf*A membrane protein gene according to the Nullarbor bioinformatics software analysis at > 90% coverage for the tetracycline-resistant gene *tet*(34*)1*. Two isolates negative for the *tet*(34)1 gene were from the Peel Forest conventional farm. However, according to the Geneious™ Pro, all 20 isolates carried a similar nucleotide sequence to *tet*(34)1 gene ORF at > 67.0% coverage and > 76.0% pairwise identity (Table 7.9). All three isolates that possessed the fosfomycin resistant gene variant, *fos*A7, were from the organic dairy farms while all isolates that showed the resistant gene variants apart from *tet*(34)1*, aph*(3), and *sul*2.2 to tetracycline, aminoglycosides, and sulphonamides respectively, were from the Peel Forest conventional dairy farm.

Table 7.2. Summary of resistant gene variants encoded by dairy farm soil *E. coli* isolates.

Based on the Nullarbor analysis

	Antimicrobial-resistant gene variant												
Isolate	***aph*(3'')-Ib_2**	***aph*(3'')-Ib_5**	***aph*(3')-Ia_3**	***aph*(6)-Id_1**	***bla*TEM-1B_1**	***fosA7*_1**	***mdf*(A)_1**	***sul*_2**	***tet*(34)_1**	***tet*(A)_6**	***tet*(B)_1**	***tet*(B)_2**	**No of resistant gene variants**
CW28A18	-	-	-	-	-	-	+	-	±	-	-	-	2
CW33S18	-	-	-	-	-	+	+	-	±	-	-	-	3
CW49A18	-	-	-	-	-	-	+	-	±	-	-	-	2
MRD21A18	-	-	-	-	-	-	+	-	±	-	-	-	2
MRD22W18	-	-	-	-	-	-	+	-	±	-	-	-	2
MRD24S17	-	-	-	-	-	-	+	-	±	-	-	-	2
PF14A18	+	-	+	+	-	-	+	+	±	-	+	-	7
PF15A18	+	-	+	+	-	-	+	+	±	-	+	-	7

± = < 90% coverage

Table 7.2 continued

Isolate	Antimicrobial-resistant gene variant													No of resistant gene variants
	aph(3'')-Ib_2	*aph*(3'')-Ib_5	*aph*(3'')-Ia_3	*aph*(3')-Id_1	*aph*(6)-1B_1	*bla*TEM-	*fosA7* _1	*mdf*(A) _1	*sul* _2	*tet*(34) _1	*tet*(A) _6	*tet*(B) _1	*tet*(B) _2	
PF25S18	-	-	-	-	-	-	+	-	±	-	-	-	2	
PF30A18	-	-	-	-	-	-	+	-	-	-	-	-	1	
PF32A18	-	-	-	-	+	-	+	-	±	+	-	-	4	
PF45W18	-	-	-	-	-	-	+	-	±	-	-	-	2	
PF52W18	-	-	-	-	-	-	+	-	-	-	-	-	1	
PF55W18	-	+	-	+	+	-	+	-	±	-	-	+	6	
TL12A18	-	-	-	-	-	-	+	-	±	-	-	-	2	
TL1S18	-	-	-	-	-	+	+	-	±	-	-	-	3	
TL2A18	-	-	-	-	-	+	+	-	±	-	-	-	3	

± = < 90% coverage

Table 7.2 continued

DfSEC Isolate	Antimicrobial-resistant gene variant												
	aph(3'')-Ib_2	*aph*(3'')-Ib_5	*aph*(3')-Ia_3	*aph*(6)-Id_1	*bla*TEM-1B_1	*fosA7* _1	*mdf*(A) _1	*sul* _2	*tet*(34) _1	*tet*(A) _6	*tet*(B) _1	*tet*(B) _2	No of resistant gene variants
TL54S18	-	-	-	-	-	-	+	-	±	-	-	-	2
TL56S18	-	-	-	-	-	-	+	-	±	-	-	-	2
TL87A18	-	-	-	-	-	-	+	-	±	-	-	-	2

± = < 90% coverage

In addition to the results obtained using the Nullarbor bioinformatics software analysis, a Geneious™ Pro query of the nucleotide sequences of ESBL genes that were investigated by Dallenne et al. (2010) (Table 7.1) were also considered in this study.

For the *bla*ACT-1 gene variant, the nucleotide sequence match between the ORF of the gene variant and the nucleotide assembly of each of the DfSEC that showed coverage > 86% with > 70% pairwise identity was queried in the NCBI protein BLAST. The results of the *bla*ACT-1 gene variant ORF nucleotide sequence protein BLAST search and that of each of the 20 DfSEC isolates were similar in encoding for the AmpC protein with the same accession number, E-value, and protein description. (Table 7.3). Relatedness of the DfSEC isolates regarding the nucleotide sequence of the ORF of the *bla*ACT-1 gene (Figure 7.2) indicates the closer relation of isolates CW28A18, MRD22W18, PF30A18, PF52W18, and TL2A18 that had matching nucleotide sequence with the ORF of the *bla*ACT-1 gene at the contig 10 but with different coordinates than the rest of the DfSEC isolates.

Table 7.3. Result of Geneious™ Pro 5.6.7 query and NCBI BLAST for *bla*ACT-1 gene variant ORF.

Gene name		**Interval**	**%Pairwise identity**	**% Query coverage**	**E value**	**Protein name**	**Accession number**
*bla*ACT-1		3-995	-	-	-	AmpC	PRK11289
DfSEC isolate	**Contig.**	**Isolate coordinates**	**%Pairwise identity**	**% Query coverage**	**E value**	**Protein name**	**Accession number**
CW28A18	10	159075-159997	71.5	84.7	3.17e-135	AmpC	PRK11289
CW33S18	49	13058-14028	71.5	84.7	3.17e-135	AmpC	PRK11289
CW49A18	38	29021-29943	70.9	84.7	5.84e-132	AmpC	PRK11289
MRD21A18	47	4752-5674	71.3	84.7	1.67e-132	AmpC	PRK11289
MRD22W18	10	13724-14646	71.5	84.7	3.12e-135	AmpC	PRK11289
MRD24S17	19	74213-75135	71.5	84.7	3.07e-135	AmpC	PRK11289
PF14A18	44	23919-24841	71.5	84.7	3.25e-135	AmpC	PRK11289
PF15A18	52	15336-16258	71.5	84.7	3.25e-135	AmpC	PRK11289
PF25S18	60	4754-5676	71.5	84.7	3.19e-135	AmpC	PRK11289

Table 7.3 continued

DfSEC isolate	Contig.	Isolate coordinates	%Pairwise identity	% Query coverage	E value	Protein name	Accession number
PF30A18	10	174201-175123	71.5	84.7	3.20e-131	AmpC	PRK11289
PF32A18	47	13058-13980	71.5	84.7	3.77e-134	AmpC	PRK11289
PF45W18	21	13613-14535	71.2	84.7	2.51e-135	AmpC	PRK11289
PF52W18	10	166671-166017	71.3	84.7	3.21e-135	AmpC	PRK11289
PF55W18	3	40899-41821	71.6	84.7	6.97e-135	AmpC	PRK11289
TL12A18	8	159275-160197	71.5	84.7	3.15e-135	AmpC	PRK11289
TL1S18	45	4753-5675	71.5	84.7	3.17e-135	AmpC	PRK11289
TL2A18	10	159185-160155	71.5	84.7	3.12e-135	AmpC	PRK11289
TL54S18	51	13058-13980	71.5	84.7	3.10e-135	AmpC	PRK11289
TL56S18	51	4754-5676	71.5	84.7	3.05e-135	AmpC	PRK11289
TL87A18	15	100742-101642	71.5	84.7	5.94e-135	AmpC	PRK11289

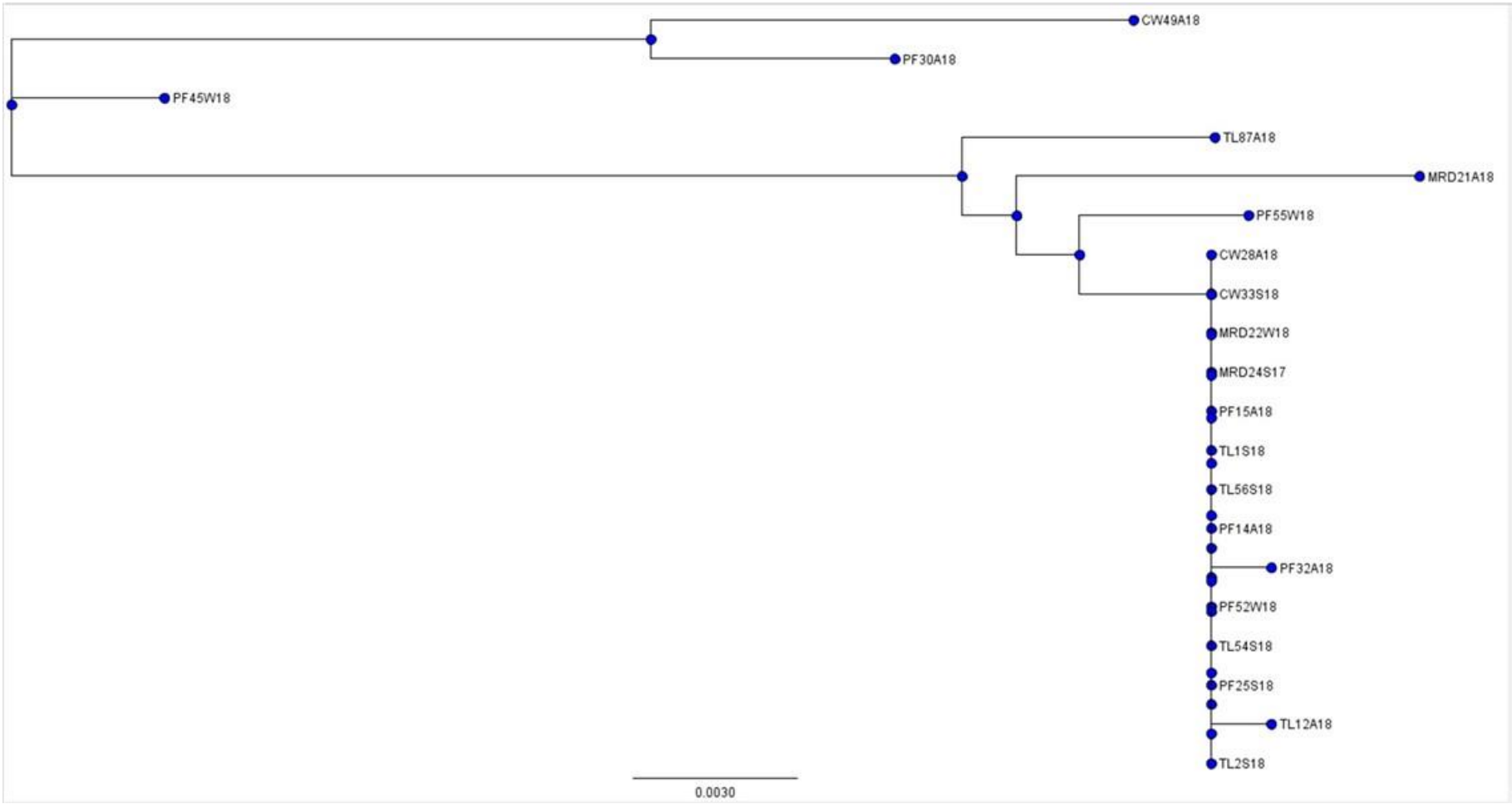

Figure 7.2 Phylogenetic tree of DfSEC isolates nucleotide match to *bla*ACT-1 gene ORF

Similar to the results obtained for the *bla*ACT-1 variant, the *bla*BIL-1 gene variant also had all the 20 DfSEC isolates carrying nucleotide sequences encoded for the production of AmpC protein of accession number PRK11289 described as beta-lactamase/D-alanine carboxypeptidase (provisional). The nucleotide sequence for both the *bla*ACT-1 and *bla*BIL-1 gene variants was located at the same contig for each isolate with similar coordinates. However, for the *bla*BIL-1 gene variant, the coverage cover was wider at > 88% coverage and > 70% pairwise identity (Table7.4). The phylogenetic tree (Figure 7.3) of the matching nucleotide sequence of the DfSEC isolates to the ORF of the *bla*BIL-1 gene, bears a close similarity to that of the *bla*ACT-1 phylogenetic tree and indicates the comparatively close relatedness of the two gene variants' (Jeong et al., 2011) nucleotide sequences at their ORFs.

Table 7.4. Result of Geneious™ Pro 5.6.7 query and NCBI BLAST for *bla*BIL-1 gene variant ORF.

Gene name		**Interval**	**%Pairwise identity**	**% Query coverage**	**E value**	**Protein name**	**Accession number**
*bla*BIL-1		82-1227	-	-	-	AmpC	PRK11289
DfSEC isolate	**Contig.**	**Isolate coordinates**	**%Pairwise identity**	**% Query coverage**	**E value**	**Protein name**	**Accession number**
CW28A18	10	159000-160084	70.9	88.9	7.02e-144	AmpC	PRK11289
CW33S18	49	12971-14055	70.9	88.9	7.02e-144	AmpC	PRK11289
CW49A18	38	28946-30030	71.2	88.9	1.38e-146	AmpC	PRK11289
MRD21A18	47	4677-5761	70.8	88.9	8.68e-143	AmpC	PRK11289
MRD22W18	10	13637-14721	70.9	88.9	6.91e-144	AmpC	PRK11289
MRD24S17	19	74138-75222	70.9	88.9	6.81e-144	AmpC	PRK11289
PF14A18	44	23844-24928	70.9	88.9	7.20e-144	AmpC	PRK11289
PF15A18	52	15261-16345	70.9	88.9	7019e-144	AmpC	PRK11289
PF25S18	60	4679-5763	70.9	88.9	7.06e-144	AmpC	PRK11289

Table 7.4 continued

DfSEC isolate	Contig.	Isolate coordinates	%Pairwise identity	% Query coverage	E value	Protein name	Accession number
PF30A18	10	174126-175210	71.3	88.9	1.15e-147	AmpC	PRK11289
PF32A18	47	12971-14055	70.9	88.9	6.86e-144	AmpC	PRK11289
PF45W18	21	13526-14611	71.4	89.0	1.70e-151	AmpC	PRK11289
PF52W18	10	165758-166842	70.9	88.9	7.11e-145	AmpC	PRK11289
PF55W18	3	40824-41908	71.0	88.9	1.54e-145	AmpC	PRK11289
TL12A18	8	159200-160284	70.9	88.9	6.98e-144	AmpC	PRK11289
TL1S18	45	4678-5762	70.9	88.9	7.02e-144	AmpC	PRK11289
TL2A18	10	159158-160242	70.9	88.9	6.91e-144	AmpC	PRK11289
TL54S18	51	12971-14055	70.9	88.9	6.88e-144	AmpC	PRK11289
TL56S18	51	4679-5763	7.09	88.9	6.75e-144	AmpC	PRK11289
TL87A18	15	100645-101729	70.9	88.9	6.82e-144	AmpC	PRK11289

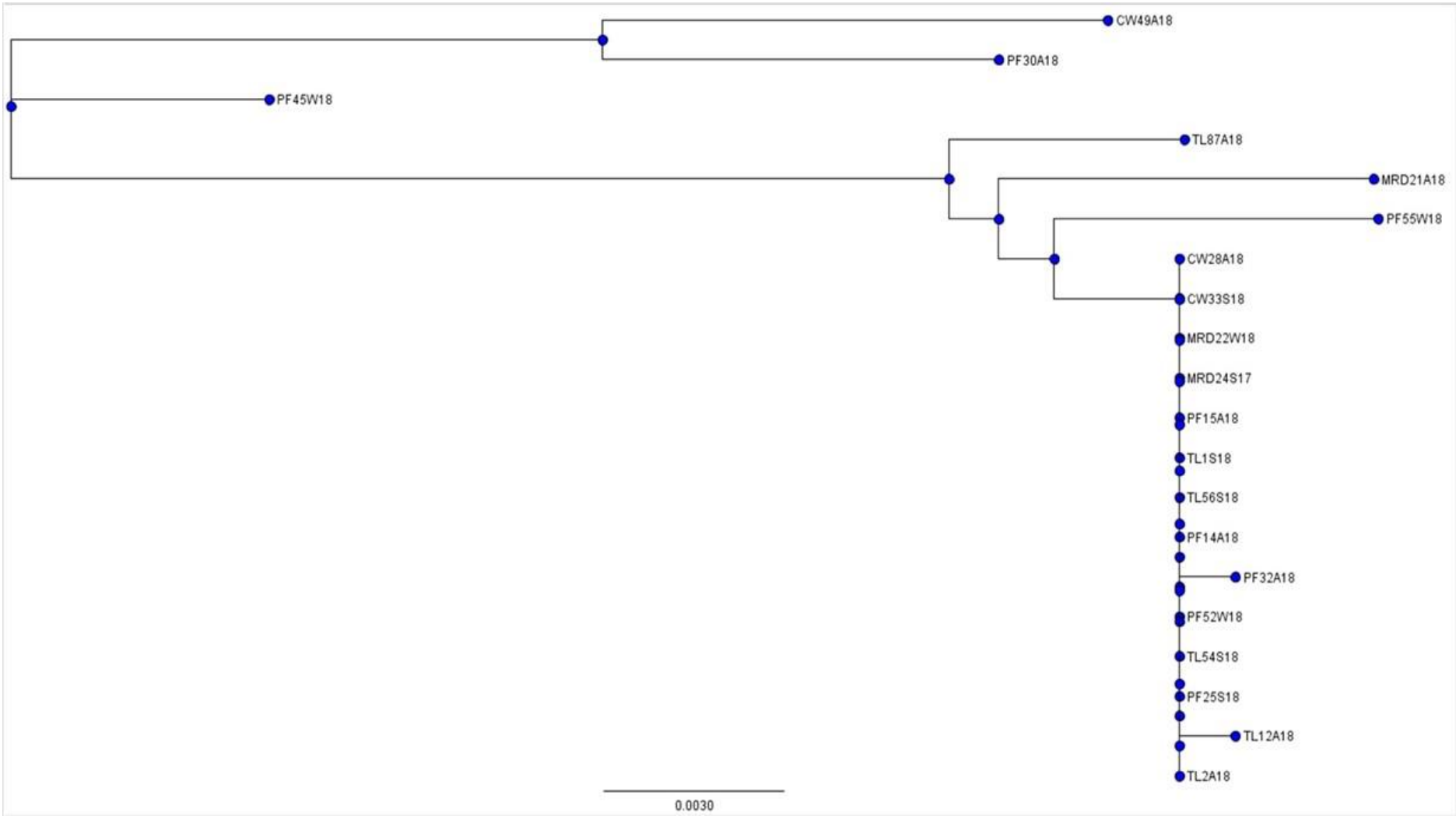

Figure 7.3 Phylogenetic tree of DfSEC isolates nucleotide match to *bla*BIL-1 gene ORF

A query of the *bla*CMY-2 gene variant's ORFs nucleotide sequence in the NCBI conserve domain resulted in an AmpC protein with the accession number PRK11289, described as a provisional version of a beta-lactamase/D-alanine carboxypeptidase enzyme. All the 20 DfSEC matching nucleotide sequences with the *bla*CMY-2 gene variant ORF encoded a protein with the same name and accession number as the *bla*CMY-2 gene variant's ORF. The *bla*CMY-2 gene variant was also located at the same contig as those of the *bla*ACT-1 and *bla*BIL-1 gene variants with similar coordinates, for each of the DfSEC isolates (Table 7.5). The phylogenetic tree of the matching nucleotide sequence of the DfSEC isolates with the *bla*CMY-2 ORF (Figure 7.4) also bears a close resemblance to the phylogenetic tree of the *bla*ACT-1 and the *bla*BIL-1 genes as the coordinates and the contigs at which matches are indicated for the various DfSEC isolates are closely matched.

Table 7.5. Result of Geneious™ Pro 5.6.7 query and NCBI BLAST for *bla*CMY-2 gene variant ORF.

Gene name		Interval	%Pairwise identity	%Query cover	E value	Protein name	Accession number
CMY-2		1-1143	-	-	-	AmpC	PRK11289
DfSEC isolate	**Contig.**	**Isolate coordinates**	**%Pairwise identity**	**% Query coverage**	**E value**	**Protein name**	**Accession number**
CW28A18	10	159000-160084	71.3	95.5	5.72e-151	AmpC	PRK11289
CW33S18	49	12971-14055	71.3	95.5	5.72e-151	AmpC	PRK11289
CW49A18	38	28946-30030	71.6	95.5	3.93e-153	AmpC	PRK11289
MRD21A18	47	4677-5761	71.2	95.5	2.47e-149	AmpC	PRK11289
MRD22W18	10	13637-14721	71.3	95.5	5.63e-151	AmpC	PRK11289
MRD24S17	19	74138-75222	71.3	95.5	5.55e-151	AmpC	PRK11289
PF14A18	44	23844-24928	71.3	95.5	5.87e-151	AmpC	PRK11289
PF15A18	52	15261-16345	71.3	95.5	5.86e-151	AmpC	PRK11289
PF25S18	60	4679-5763	71.3	95.5	5.75e-151	AmpC	PRK11289
PF30A18	10	174126-175210	71.7	95.5	9.35e-155	AmpC	PRK11289
PF32A18	47	12971-14055	71.3	95.5	5.60e-151	AmpC	PRK11289
PF45W18	21	13526-1411	71.8	95.5	4.63e-158	AmpC	PRK11289
PF52W18	10	165758-166842	71.3	95.5	5.80e-151	AmpC	PRK11289
PF55W18	3	40824-41908	71.4	95.5	4.39e-152	AmpC	PRK11289

Table 7.5 continued

DfSEC isolate	Contig.	Isolate coordinates	%Pairwise identity	% Query coverage	E value	Protein name	Accession number
TL12A18	8	159200-160284	71.3	95.5	5.69e-151	AmpC	PRK11289
TL1S18	45	4678-5762	71.3	95.5	5.72e-151	AmpC	PRK11289
TL2A18	10	159158-160242	71.3	95.5	5.64e-151	AmpC	PRK11289
TL54S18	51	12971-14055	71.3	95.5	5.61e-151	AmpC	PRK11289
TL56S18	51	4679-5763	71.3	95.5	5.51e-151	AmpC	PRK11289
TL87A18	15	100645-101729	71.3	95.5	5.56e-151	AmpC	PRK11289

Al20 DfSEC isolates encoded for the same protein (enzyme) as the ORF of the *bla*CMY-2 gene variant.

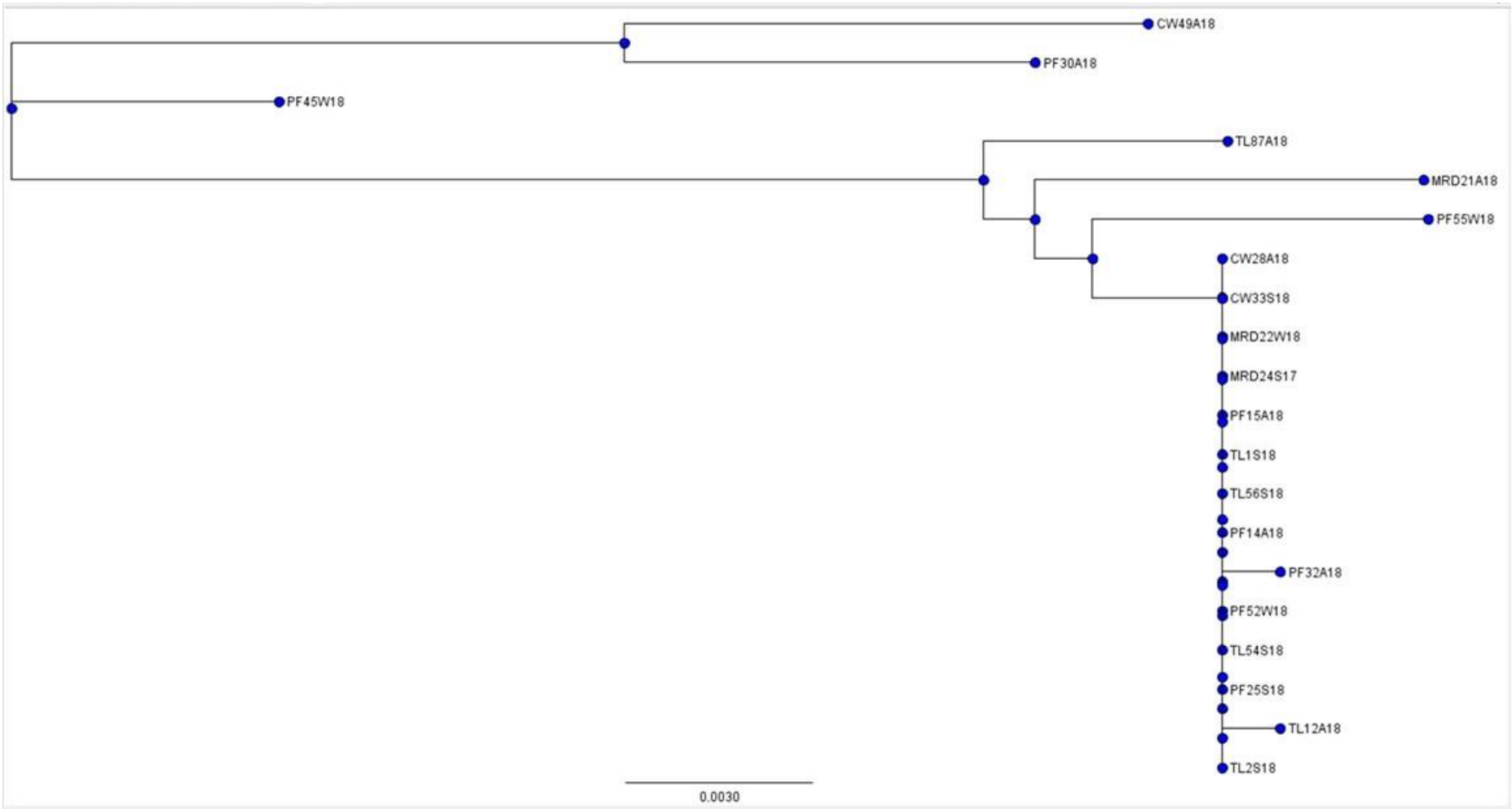

Figure 7.4 Phylogenetic tree of DfSEC isolates nucleotide match to *bla*CMY-2 gene ORF

Table 7.6. Result of Geneious™ Pro 5.6.7 query and NCBI BLAST for *bla*DHA-1 gene variant ORF.

Gene name	Interval	%Pairwise identity	%Query coverage	E value	Protein name	Accession number
*bla*DHA-1	113-1109	-	-	-	AmpC	PRK11289

DfSEC isolate	Contig.	Isolate coordinates	%Pairwise identity	% Query coverage	E value	Protein name	Accession number
CW49A18	38	28966-29975	63.1	88.3	1.79e-24	AmpC	PRK11289
PF30A18	10	174145-175155	62.9	88.4	2.68e-22	AmpC	PRK11289

Of the 20 DfSEC isolates only CW49A18 and PF30A18 had nucleotide sequences with enough coverage (88.33% and 88.42%) and pairwise identity (63.1% and 62.9%) respectively, to encode for proteins from their queries. The protein encoded for by the *bla*DHA-1 gene variant ORF was an AmpC enzyme with accession number PRK11289, similar to the protein CW49A18 and PF30A18 encoded for (Table 7.6).

Table 7.7. Result of Geneious™ Pro 5.6.7 query and NCBI BLAST for *bla*MIR-1 gene variant ORF.

Gene name		**Interval**	**%Pairwise identity**	**%Query coverage**	**E value**	**Protein**	**Accession number**
*bla*MIR-1 ORF	1-1143	1-1143	-	-	-	AmpC	PRK11289
DfSEC isolate	**Contig.**	**Gene coordinates**	**%Pairwise identity**	**%Query coverage**	**E value**	**Protein**	**Accession number**
CW28A18	10	159997-158984	70.5	88.5	3.62e-128	AmpC	PRK11289
CW33S18	49	13058-14071	70.5	88.5	3.61e-128	AmpC	PRK11289
CW49A18	38	29943-28930	70.1	88.5	1.21e-121	AmpC	PRK11289
MRD21A18	47	5674-4661	70.1	88.5	9.85e-123	AmpC	PRK11289
MRD22W18	10	13724-14737	70.5	88.5	3.56e-128	AmpC	PRK11289
MRD24S17	19	75135-74122	70.5	88.5	3.51e-128	AmpC	PRK11289
PF14A18	44	24841-23823	70.5	88.5	3.71e-128	AmpC	PRK11289
PF15A18	52	16258-15245	70.5	88.5	3.70e-128	AmpC	PRK11289
PF25S18	60	5676-4663	70.5	88.5	3.63e-128	AmpC	PRK11289
PF30A18	10	175123-174110	70.2	88.5	1.22e-121	AmpC	PRK11289

Table 7.7 continued4.31

DfSEC isolate	Contig.	Gene coordinates	%Pairwise identity	%Query coverage	E value	Protein	Accession number
PF32A18	47	13058-14071	70.2	88.5	4.31e-127	AmpC	PRK11289
PF45W18	21	13613-14626	70.5	88.5	3.48e-128	AmpC	PRK11289
PF52W18	10	166755-165742	70.5	88.5	3.66e-128	AmpC	PRK11289
PF55W18	3	41821-40808	70.5	88.5	3.38e-128	AmpC	PRK11289
TL12A18	8	160197-159184	70.5	88.5	3.59e-128	AmpC	PRK11289
TL1S18	45	5675-4662	70.5	88.5	3.61e-128	AmpC	PRK11289
TL2S18	10	160155-159142	70.5	88.5	3.56e-128	AmpC	PRK11289
TL54S18	51	13058-14071	70.5	88.5	3.54e-128	AmpC	PRK11289
TL56S18	51	5676-4663	70.5	88.5	3.48e-128	AmpC	PRK11289
TL87A18	15	101642-100629	70.5	88.5	3.51e-128	AmpC	PRK11289

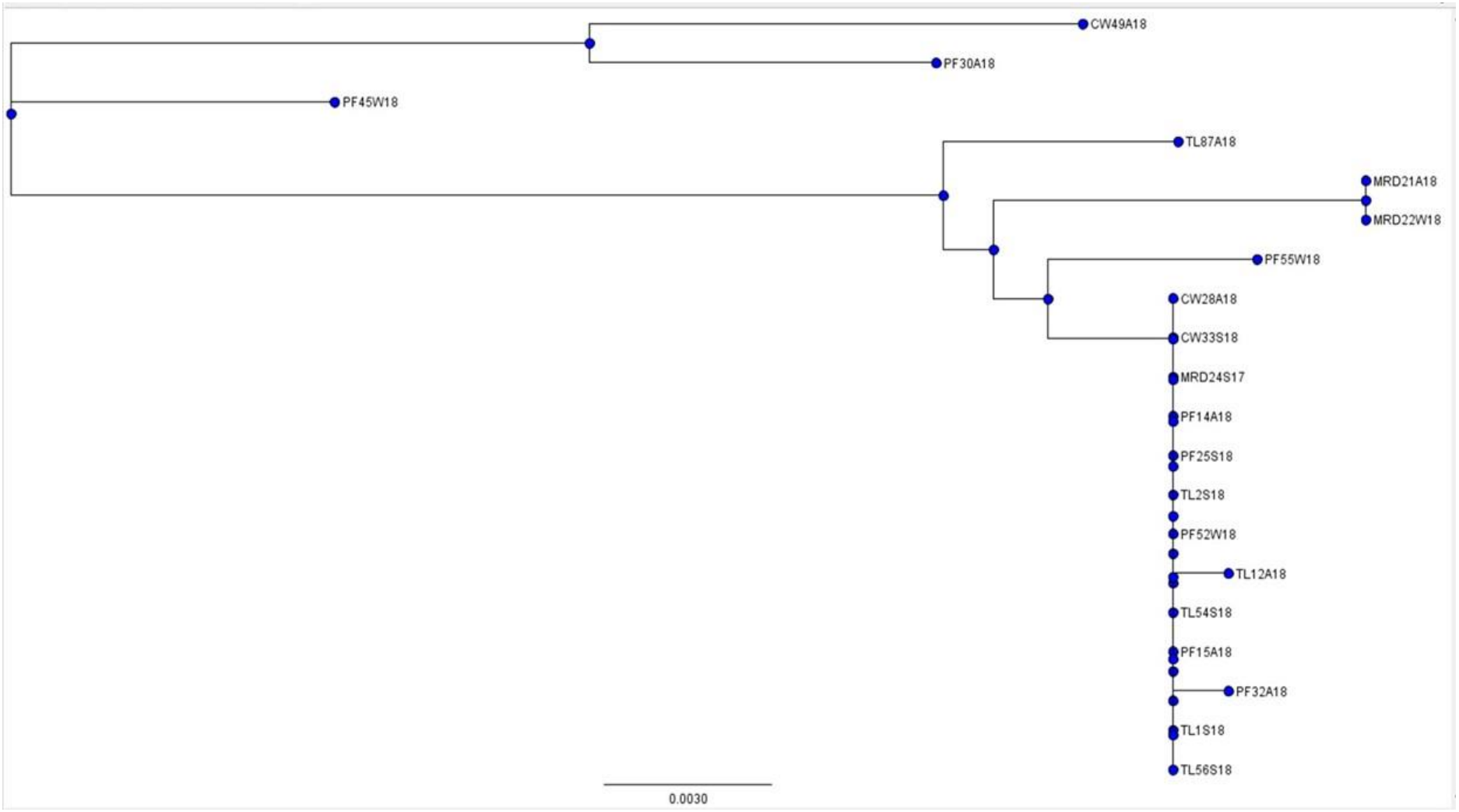

Figure 7.5 Phylogenetic tree of DfSEC isolates nucleotide match to *bla*MIR-1 gene ORF

In this study, the *bla*ACT-1, *bla*BIL-1, *bla*CMY-2 and the *bla*MIR-1 gene variants ORF had similar nucleotide matches with the 20 DfSEC isolates. The nucleotide matches were at the same contig and location (Tables 7.3, 7.4, 7.5, and 7.6) inferring the possibility that an isolate may encode for either *bla*ACT-1, *bla*BIL-1, *bla*CMY-2 or the blaMIR-1 gene variant only and not all four of them according to the parameters used for the Geneious™ Pro phylogenetic trees with a gap penalty of 12 (Figures 7.2, 7.3, 7.4 and 7.5).

Table 7.8 Location of the matched nucleotide sequence for *bla*ACT-1, *bla*BIL-1, *bla*CMY-2, and *bla*MIR-1 gene ORF with DfSEC isolates.

According to Geneious™ Pro analysis.

	bla gene ORF nucleotide sequence match coordinates			
DfSEC	*bla*ACT-1	*bla*BIL-1	*bla*CMY-2	*bla*MIR-1
CW28A18	159075-159997	159000-160084	159000-160084	159997-158984
CW33S18	13058-14028	12971-14055	12971-14055	13058-14071
CW49A18	29021-29943	28946-30030	28946-30030	29943-28930
MRD21A18	4752-5674	4677-5761	4677-5761	5674-4661
MRD22W18	13724-14646	13637-14721	13637-14721	13724-14737
MRD24S17	74213-75135	74138-75222	74138-75222	75135-74122
PF14A18	23919-24841	23844-24928	23844-24928	24841-23823
PF15A18	15336-16258	15261-16345	15261-16345	16258-15245
PF25S18	4754-5676	4679-5763	4679-5763	5676-4663
PF30A18	174201-175123	174126-175210	174126-175210	175123-174110
PF32A18	13058-13980	12971-14055	12971-14055	13058-14071
PF45W18	13613-14535	13526-14611	13526-1411	13613-14626
PF52W18	166671-166017	165758-166842	165758-166842	166755-165742
PF55W18	40899-41821	40824-41908	40824-41908	41821-40808

Table 7.7 continued

	bla gene ORF nucleotide sequence match coordinates			
DfSEC	*bla*ACT-1	*bla*BIL-1	*bla*CMY-2	*bla*MIR-1
TL12A18	159275-160197	159200-160284	159200-160284	160197-159184
TL1S18	4753-5675	4678-5762	4678-5762	5675-4662
TL2A18	159185-160155	159158-160242	159158-160242	160155-159142
TL54S18	13058-13980	12971-14055	12971-14055	13058-14071
TL56S18	4754-5676	4679-5763	4679-5763	5676-4663
TL87A18	100742-101642	100645-101729	100645-101729	101642-100629

Table 7.9. Result of Geneious™ Pro 5.6.7 query and NCBI BLAST for *bla*TEM-1 gene variant ORF.

Gene name		**Interval**	**%Pairwise identity**	**%Query coverage**	**E value**	**Protein name**	**Accession number**
*bla*TEM-1		7-858	-	-	-	PRK15442	PRK15442
DfSEC isolate	**Contig.**	**Gene coordinates**	**%Pairwise identity**	**%Query cover**	**E value**	**Protein**	**Accession number**
PF32A18	22	57411-58271	99.9	100	0e+00	PRK15442	PRK15442
PF55W18	68	3663-4523	99.9	100	0e+00	PRK15442	PRK15442

The *bla*MIR-1 gene variant had 20 DfSEC's with an 88.5% query cover and > 70% pairwise identity wide coverage percentage compared to the *bla*ACT-1, *bla*BIL-1, and *bla*CMY-2 but at the same contig and similar coordinates and the nucleotide sequence encoding for the same proteins with the same accession number and description of β-lactamase/D-alanine carboxypeptidase (Table 7.8).

The DfSEC isolates PF32A18 and PF55W18 had coverage of 100% and 99.9% pairwise identity with the *bla*TEM-1 gene variant and the nucleotide sequence query indicated the production of the *bla*TEM-1 β-lactamase hydrolysing enzyme from the Peel Forest conventional dairy farm, isolated in autumn of 2018 encode for the production of *bla*TEM-1 ESBL enzyme (Table7.8) described as beta-lactamase at the NCBI protein domain site (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

For the *tet*(34)1 tetracycline resistance genes, the query cover threshold of > 67% coverage cover and > 75% pairwise identity was used during the Geneious™ Pro 5.6.7 analysis. The gene's accession number of AB061440.1 was sourced from a peer-reviewed journal article by Roberts (2005). The *tet*(34)1 ORF nucleotide sequence was queried in the NCBI conserved domain protein BLAST and the result corresponded to a protein with the same name as the accession number of PRK09177 which was described as a validated xanthine-guanine phosphoribosyltransferase. Subsequently, the nucleotide sequence at the matching coordinates at the designated contig on the genome assembly of the DfSEC was queried in the NCBI protein BLAST for the possible proteins that the isolates may encode. All 20 DfSEC isolates carried nucleotide sequences that encoded for similar protein to the protein encoded by the nucleotide sequence of the *tet*(34)1 gene ORF (Table 7.9).

Table 7.10. Result of Geneious™ Pro 5.6.7 query and NCBI BLAST for *tet*(34)1 gene variant.

Gene name		**Interval**	**%Pairwise identity**	**%Query cover**	**E value**	**Protein**	**Accession number**
tet(34)1 ORF		7-465	-	-	-	PRK09177	PRK09177
DfSEC isolate	**Contig.**	**Gene coordinates**	**%Pairwise identity**	**%Query cover**	**E value**	**Protein name**	**Accession number**
CW28A18	26	39910-40265	75.6	67.3	5.34e-65	PRK09177	PRK09177
CW33S18	16	102168-102523	75.0	67.3	2.76e-62	PRK09177	PRK09177
CW49A18	50	9363-9722	75.1	67.3	6.63e-64	PRK09177	PRK09177
MRD21A18	28	16951-17306	75.1	67.3	2.81e-62	PRK09177	PRK09177
MRD22W18	36	7614-7969	75.1	67.3	2.72e-62	PRK09177	PRK09177
MRD24S17	1	16888-17243	75.6	67.3	5.18e-65	PRK09177	PRK09177
PF14A18	7	127893-128248	75.4	67.3	6.67e-64	PRK09177	PRK09177
PF15A18	7	127893-128248	75.4	67.3	6.66e-64	PRK09177	PRK09177
PF25S18	9	122050-122405	75.1	67.3	2.78e-62	PRK09177	PRK09177
PF30A18	14	114059-114414	74.5	67.3	4.80e-59	PRK09177	PRK09177

Table 7.9 continued

DfSEC isolate	Contig.	Gene coordinates	%Pairwise identity	%Query cover	E value	Protein name	Accession number
PF32A18	20	16882-17237	75.1	67.3	2.70e-62	PRK09177	PRK09177
PF45W18	49	17000-17355	75.4	67.3	6.27e-64	PRK09177	PRK09177
PF52W18	46	16882-17237	74.8	67.3	3.41e-61	PRK09177	PRK09177
PF55W18	9	90304-90659	75.1	67.3	2.59e-52	PRK09177	PRK09177
TL12A18	30	51909-52264	75.4	67.3	6.46e-64	PRK09177	PRK09177
TL1S18	17	16882-17237	75.1	67.3	2.70e-62	PRK09177	PRK09177
TL2S18	46	8589-8944	75.4	67.3	6.40e-64	PRK09177	PRK09177
TL54S18	30	19512-19867	75.1	67.3	2.71e-62	PRK09177	PRK09177
TL56S18	2	241997-242352	75.1	67.3	2.66e-62	PRK09177	PRK09177
TL87A18	32	16984-17339	75.1	67.3	6.32e-64	PRK09177	PRK09177

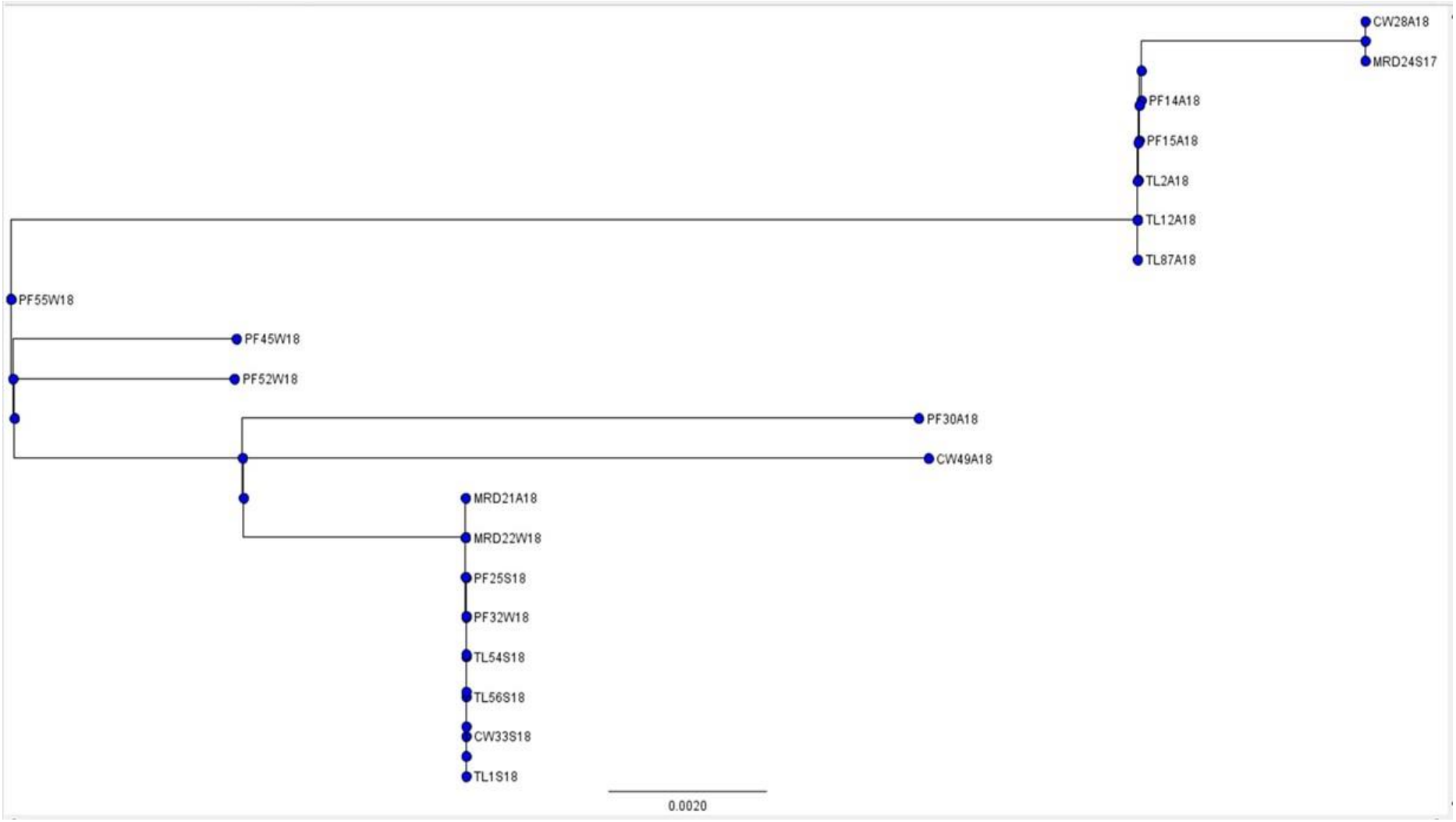

Figure 7.6 Phylogenetic tree of DfSEC isolates nucleotide match to *tet*(34)1 gene ORF

***Tet*(34)1 gene variant ORF nucleotide sequence.**

TTACTTTTGCTTGCGATTAATTGGTTCCACAAATTGCACTGTGGTATCCCATGGCTGCTCAATCCATGTATCTTG
AGCAATATCAACCACGTAATCATCAAGAAGAGTGGCACCTGATGGCTTAGCGCAAACAGCAATCAGTTTCGCT
TTCGGGTACATTTCACGTAGTTTACGCGCTGTATCACCGCTATCCACTAGGTCTTCGACGATTAGGTAACCTTCG
CCATCACCTTCTGGCGCTTTTAGCACGGTCATATCACGCTGGTGATCATGATCGTAGCTAGAGATACAAATGGT
ATCAACGTGACGAATACCAAGTTCACGCGCAAGAATAGCACCCGGTACTAGACCGCCACGGCTCACCGCCCAA
ATACCTTTCCACTGTTCAGCTGGCATCTGTTTTTCAGCAAGCTGACGACAGTAATTTTGCATGGCGTCCCAAGTG
ATAATGAATTTTTTGCTCATTGTAATAACCTAATAATTTTGAAGTTTGCATCCCCGTATTAGGCAA

DfSEC matching nucleotide sequence with *tet*(34)1 gene variant ORF of:

TATCCCACGGCTGTTCGATCCAGGTATCTTGCGGGATATCAACAACATAGTCATCAACCAGCGGACGACCAGC
CGGTTTTGCGAAGATAGTCACAAAATGTGCTTTTGGATACATTTCACGAATCGCAACCGCAGTACCACCGGTAT
CCACCAGGTCATCAATAACGATGAAGCCTTCGCCATCGCCTTCTGCGCGTTTTAGCACTTTAAGCTCGCGCTGG
TTGTCGTGATCGTAGCTGGAAATACAAACGGTATCGACATGACGAATACCCAGTTCACGCGCCAGTAACGCAC
CCGGTACCAGACCGCCACGGCTTACGGCAATAATGCCTTTCCATTGTTCAGAAGGCATCAGT

or

Similarly, DfSEC isolates had matching nucleotide sequence with the *tet*(34)1 gene variant ORF
ACTGATGCCTTCTGAACAATGGAAAGGCATTATTGCCGTAAGCCGTGGCGGTCTGGTACCGGGTGCGTTACTG
GCGCGTGAACTGGGTATTCGTCATGTCGATACCGTTTGTATTTCCAGCTACGATCACGACAACCAGCGCGAGCT
TAAAGTGCTGAAACGCGCAGAAGGCGATGGCGAAGGCTTCATCGTTATTGATGACCTGGTGGATACCGGTGG
TACTGCGGTTGCGATTCGTGAAATGTATCCAAAAGCGCACTTTGTCACCATCTTTGCAAAACCGGCTGGTCGTC
CGCTGGTTGATGATTATGTTGTTGATATCCCGCAAGATACCTGGATCGAACAGCCGTGGGATA

All the above nucleotide sequences encoded for the validated protein xanthine-guanine phosphoribosyltransferase with similar accession numbers and intervals from 2-355 as the previous nucleotide sequence. The protein PRK09177 is a member of the protein superfamily cl00306. This

family encompasses 41 other proteins that are involved in metabolic activities of *E. coli* amino acid transformations (https://www.ncbi.nlm.nih.gov/Structure/cdd/cl00309).

The combination of the resistance genes encoded by the 20 DfSEC that were sequenced for genomic analysis by the Nullarbor and Geneious™Pro5.6.7 bioinformatics software are shown in Table 7.10. A phylogenetic of the nucleotide match of the *tet*(34)1 gene ORF with the DfSEC isolates (Figure 7.6) indicated close relatedness of isolates from different farms. Similarly, the close relatedness of the *bla*ACT-1, *bla*BIL-1, *bla*CMY-2, and the *bla*MIR-1 gene variants compared to the *tet*(34)1 gene is indicated by the cluster of the *bla*ACT-1, *bla*BIL-1, *bla*CMY-2, and the *bla*MIR-1 gene variants genes ORF nucleotide matches as opposed to the *tet*(34)1 gene match as shown in the phylogeny tree with the two exemplar isolates (Figure 7.7).

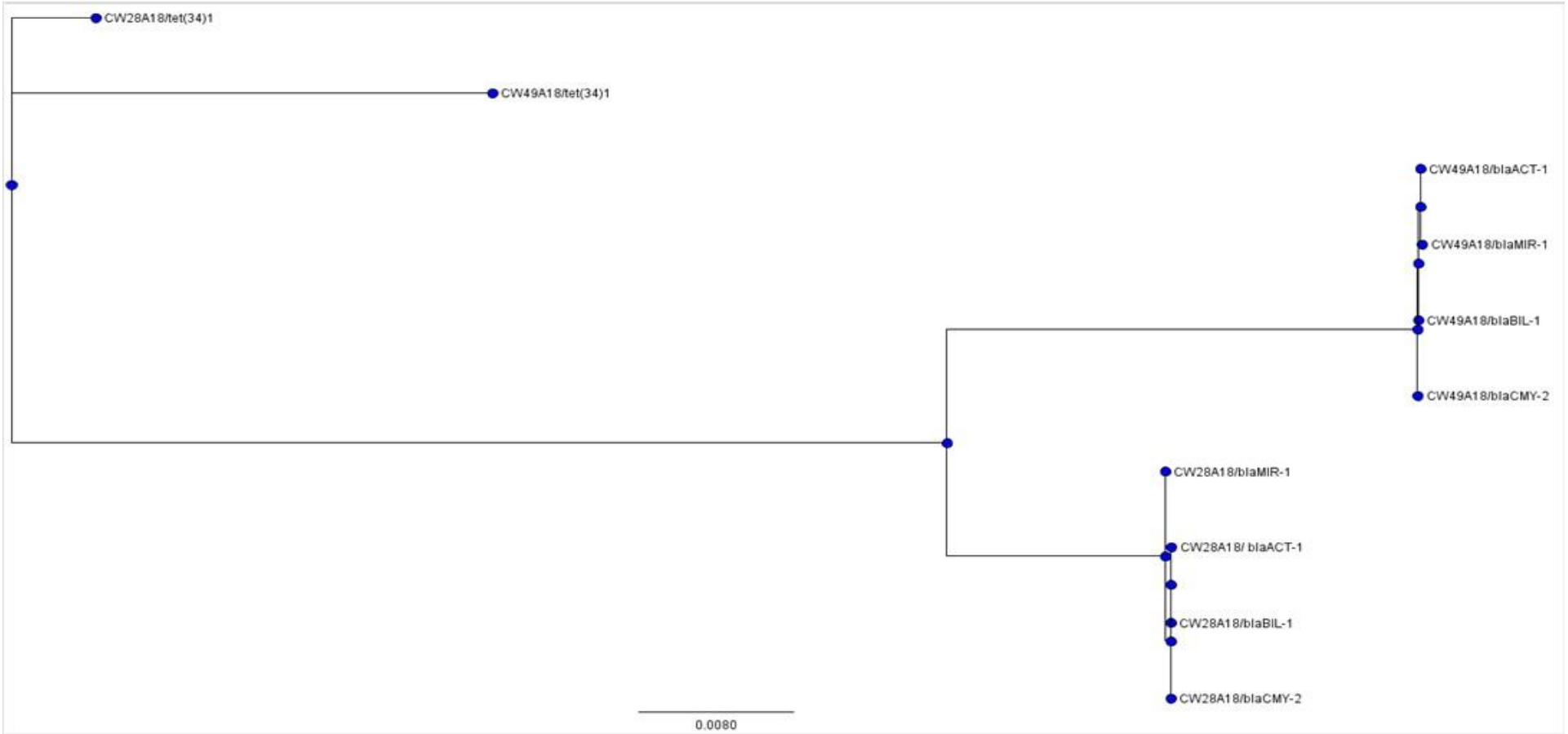

Figure 7.7 Phylogenetic tree of *bla*ACT-1, *bla*BIL-1, *bla*CMY-2, *bla* MIR-1, and *tet*(34)1 gene ORF with two exemplar DfSEC isolates.

Indicating a close relationship between these *bla* genes compared to tetracycline (*tet*) gene variant's ORF nucleotide sequence (gap penalty of 6 in Geneious Pro).

Table 7.11. Summary of antimicrobial resistance genes/matched nucleotide sequence encoded by DfSEC isolates

DfSEC isolate	*bla*ACT-1	*bla*BIL-1	*bla*CMY-2	*bla*DHA-1	*bla*MIR-1	*bla*TEM	*tet*(34)1	*aph*3''-Ib_2	*aph*3''-Ib_5	*aph*3'-Ia_3	*aph*6-Id_1	*tet*(A6)	*tet*(B1)	*tet*(B2)	fosA7.1	*Mdf*.A1	*sul* (2.2)	*bla*TEM	*tet*(34)1
	Geneious™ Pro Analysis							Nullarbor analysis											
CW28A18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
CW33S18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	+	-	-	±
CW49A18	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
MRS21A18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
MRD22W18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
MRD24S17	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
PF14A18	+	+	+	-	+	-	+	+	-	+	+	-	+	-	-	+	+	-	±
PF15A18	+	+	+	-	+	-	+	+	-	+	+	-	+	-	-	+	+	-	±

aph=aminoglycoside; *bla*ACT, *bla*BIL, *bla*CMY, *bla*DHA, *bla*TEM, *tet*=tetracyclines; *fos*=fosfomycin: *mdf*=multidrug transporter: *sul*=sulphonamide

Table 7.10 continued

DfSEC isolate	*bla*ACT-1	*bla*BIL-1	*bla*CMY-2	*bla*DHA-1	*bla*MIR-1	*bla*TEM	*tet*(34)1	*aph*3''-Ib_2	*aph*3''-Ib_5	*aph*3'-Ia_3	*aph*6-Id_1	*tet*(A6)	*tet*(B1)	*tet*(B2)	fosA7.1	*Mdf*.A1	*sul* (2.2)	*bla*TEM	*tet*(34)1
	Geneious™ Pro analysis							**Nullarbor analysis**											
PF25S18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
PF30A18	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
PF32A18	+	+	+	-	+	+	+	-	-	-	-	+	-	-	-	+	-	+	±
PF45W18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
PF52W18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
PF55W18	+	+	+	-	+	+	+	-	+	-	+	-	-	+	-	+	-	+	±
TL12A18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
TL1S18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	+	-	-	±

aph=aminoglycoside; *bla*ACT, *bla*BIL, *bla*CMY, *bla*DHA, *bla*TEM, *tet*=tetracyclines; *fos*=fosfomycin: *mdf*=multidrug transporter: *sul*=sulphonamide

Table 7.10 continued

DfSEC isolate	*bla*ACT-1	*bla*BIL-1	*bla*CMY-2	*bla*DHA-1	*bla*MIR-1	*bla*TEM	*tet*(34)1	*aph*3''-Ib_2	*aph*3''-Ib_5	*aph*3'-Ia_3	*aph*6-Id_1	*tet*(A6)	*tet*(B1)	*tet*(B2)	fosA7.1	*Mdf*.A1	*sul* (2.2)	*bla*TEM	*tet*(34)1
	Geneious™ pro analysis							**Nullarbor analysis**											
TL2A18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	+	+	-	-	±
TL54S18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
TL56S18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±
TL87A18	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	±

aph=aminoglycoside; *bla*ACT, *bla*BIL, *bla*CMY, *bla*DHA, *bla*TEM, *tet*=tetracyclines; *fos*=fosfomycin: *mdf*=multidrug transporter: *sul*=sulphonamide

Differences in parameter tuning and algorithm mechanisms may cause different clustering software tools to produce different results based mainly on the way the software program was programmed to identify and remove redundant data (Zou, Lin, Jiang, Liu, & Zeng, 2018). The volume of short reads, unique versus non-unique mapping, and base quality, during sequence alignment of experimental sample and reference sample genome, are some steps that may carry errors and cause different outputs (Dolled-Filhart, Lee, Ou-yang, Haraksingh, & Lin, 2013). These are all tied up in the type of assembler (EULER-SR, Velvet, IDBA-UD, or SPAdes) and the Phred-Phrad consensus package adopted by the assembler (Cameron et al., 2017), and these may affect the output the software provides (Zhang et al., 2011). The differences between how Geneious™ Pro 5.6.7 and Nullarbor dealt with gaps and anomalies in the nucleotide sequence of the isolates as well as the coverage and pairwise percentages adopted may have resulted in the differences in their outputs (Pedersen & Quinlan, 2017). The ResFinder for determination of resistance genes encoded by bacteria species used for the Nullarbor analysis set the coverage range at 2/5 of the length of the resistant gene with 98% pairwise identity (Zankari et al., 2012). For the Geneious™ Pro 5.6.7 analysis, the coverage was set at > 80% and pairwise identity at > 70% similarities between DfSEC sequence assembly and nucleotide sequence of resistant gene ORF. For the *tet*(34)1 gene, however, the threshold for the Geneious™ Pro 5.6.7 in this study was reduced to > 67% coverage cover and > 75% pairwise identity. The set parameters, although within acceptable limits as indicated by (Devos & Valencia, 2000) were lower than that set for the Nullarbor analysis (Chapter 7.1) and may explain the discrepancies between the two outputs.

In this study, the resulting outputs of both the Nullarbor and Geneious™ Pro 5.6.7 bioinformatics software analysis (Table 7.10) indicated that the combined isolates from the conventional dairy farm encoded nucleotide sequence with > 84% coverage and > 74% pairwise identity to the ORF of more resistant gene variants than the combined isolates from the organic dairy farms. The isolates PF14A18 and PF15A18 carried the most resistant gene variants, of 7/12 apiece according to the Nullarbor analysis. These were followed by PF55W18 that bore 6/12 gene variants. The CW49A18, CW33S18, and PF30A18 with 3/12 resistant gene variants were the third highest. Similarly, the DFSEC

isolates from the Peel Forest conventional dairy farm had three of its isolates PF55W18, PF30A18, and PF32A18 having 4/7 nucleotide matches to the ORFs of the genes analysed by the Geneious™ Pro software. DfSEC isolates that encoded nucleotide sequence matches for the *bla*ACT-1, *bla*BIL-1, *bla*CMY-2, or the *bla*MIR-1 gene counted at a single gene variant match. Only CW49A18 from the Clearwaters organic dairy farm had 4/7 matched nucleotide sequences to the ORFs of these gene variants. The rest of the isolates each had 3/7 gene ORF nucleotide sequence matches (Table 7.10).

By the use of whole-genome multi-locus sequence typing (wgMLST) of *E. coli* BioNumerics (http://www.applied-maths.com/sites/default/files/extra/Release-Note-Eschericha-coli-Shigella-schema.pdf., a minimum spanning tree was created showing the loci differences between all 20 DfSEC isolates from the four farms that showed multi-resistance to antimicrobials (Figure 7.8).

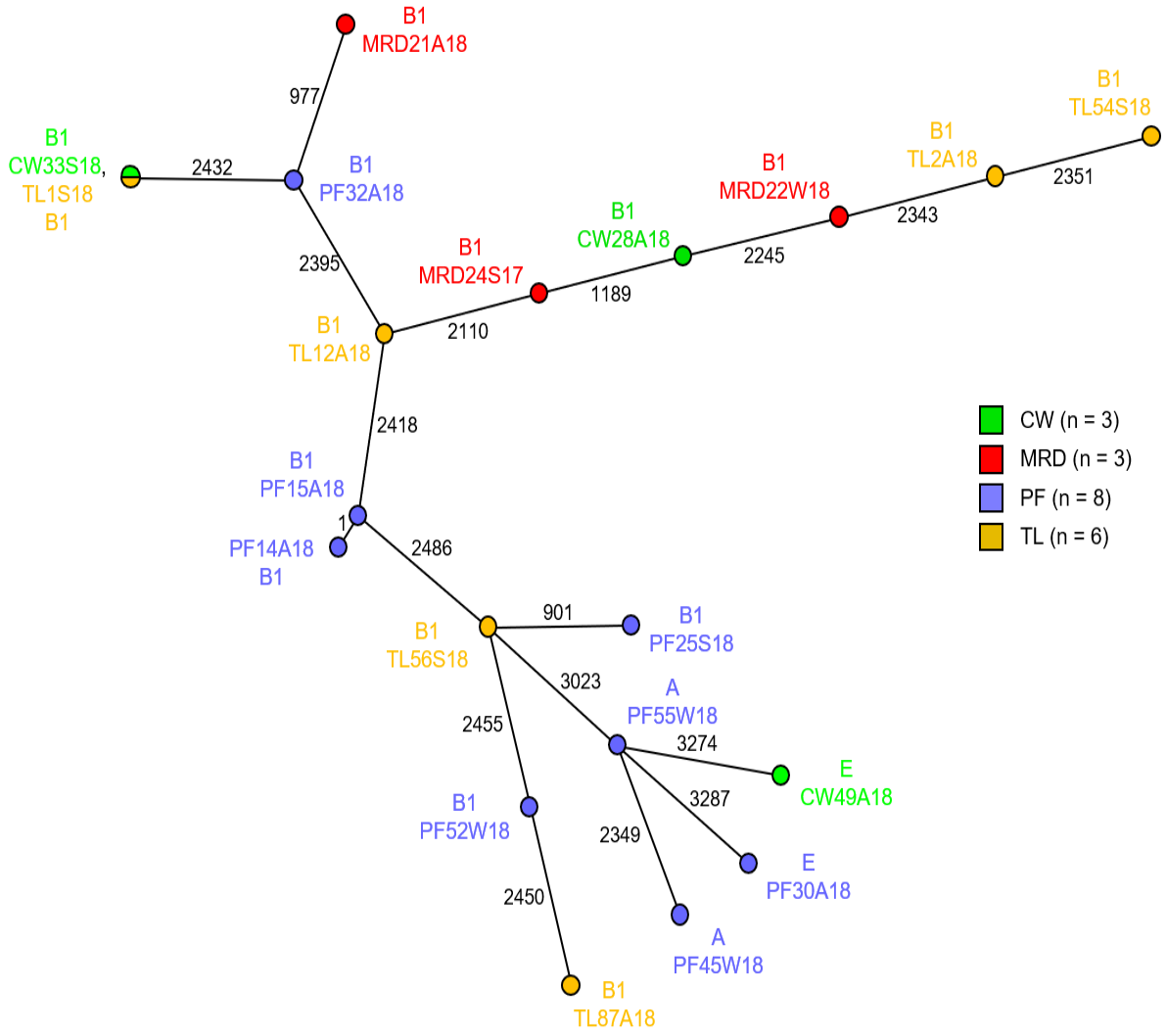

Figure 7.8. Minimum spanning tree of DfSEC isolates with multiple antimicrobial resistance

CW=Clearwaters organic farm: PF=Peel Forest conventional farm: MRD=Mill Road conventional dairy farm TL=Totara Valley organic dairy farm

S17=spring 2017: S18=spring 2018: A18=autumn 2018: W18=winter 2018

DfSEC isolates CW33S18 and TL1S18 were the closest related isolates of the B1 phylogenetic group with no loci differences by wgMLST (Figure 7.8); potentially, an indication of a common source of origin. The B1 phylogenetic group members of PF14A18 and PF15A18 were the next close relatives with a 1 locus difference. These two organisms were isolated from the same paddock at the same sampling time and may explain their close relations.

Two of the isolates from the Peel Forest conventional dairy farm were of the phylogenetic group A and were comparatively distant from the isolates of the B1 group discussed above which may indicate differences in origin at 2349 loci differences apart. The two DfSEC isolates of the phylogenetic group E, one each from the geographically closely located Clearwaters and Peel Forest farms (Chapter 3), were also comparatively distantly related by loci difference of 3287 and 3274 signifying the possibility of differences in origin.

From a summary of the BioNumerics bioinformatics software analysis (https://www.applied-maths.com/news/e-coli-genotyping-plugin-version-12-available) (Table 7.11), both isolates of the phylogenetic group E (CW49A18 and PF30A18) indicated the same virulence determinants of *iss*, *air*, *gad*, and *eiLA*, and the phylogenetic group A (PF45W18 and PF55W18) isolates carried a single virulence factor, *iss* with all four closer in relation compared to the rest of the isolates (Figure 7.8). No virulence determinants were detected in TL87A18, only one virulence determinant was detected in PF45W18, and multiple virulence determinants in the remaining 18. The type 1 fimbriae, *fim*H gene alleles, were found in all 20 isolates but differed in the variant they each carried. The *fim*H2 allele, however, was found in CW33A18, TL1S18, and TL54S18. The *fim*H8 allele was detected in MRD24S17 and TL12A18. The two isolates of the phylogenetic group A PF55W18 and PF45W18 carried the multi-resistant *fim*H30 allele. The rest of the DfSEC isolates (n=13) carried other less common variants of the *fim*H gene variants as per work carried out by Roer et al. (2017).

Table 7.12 Phylogenetic grouping of DfSEC isolates based on Clermont *et al*. (2013) protocol with multiple resistance genes; serotype and virulence determinants according to BioNumerics bioinformatics software analysis.

Farm	Isolate	Resistant type	Phylogenetic group	Serotype		Virulence determinant
Clearwaters (CW)	**28-A18**	ESBL+AmpC[#]	B1	O10	:H42	*iss, IpFA*
	33-S18	ESBL+AmpC[#]	B1	-	:H2	*iss, IpFA*
	49-A18	ESBL+AmpC[#]	E	O11	:H15	*iss, air, gad, eiLA*
Mill Road (MRD)	**21-A18**	ESBL+AmpC[#]	B1	O45	:H51	*iss, IpFA*
	22-W18	ESBL+AmpC[#]	B1	O166	:H49	*iss, IpFA*
	24-S17	ESBL+AmpC[#]	B1	O150	:H8, H40	*IpFA*
Peel Forest (PF)	**14-A18**	ESBL+AmpC[#]	B1	O9	:H21	*iss, IpFA, iha*
	15-A18	ESBL+AmpC[#]	B1	O9	:H21	*iss, IpFA, iha*
	25-S18	ESBL+AmpC[#]	B1	-	:H16	*iss,*
	30-A18	ESBL+AmpC[#]	E	O138	:H48	*iss, air, gad, eiLA*

air=enteroaggregative immunoglobulin protein: *astA*=heat stable enterotoxin 1: *cdtb*=cytolethal distending toxin B

eiLA=Salmonella HilA homolog: *gad*=glutamate decarboxylase: *iha*=adherence protein: *iss*=increased serum survival: *IpFA*=long polar fimbriae

Table 7.10 continued

Farm	Isolate	Resistant type	Phylogenetic group	Serotype		Virulence determinant
Peel Forest	**32-A18**	ESBL+AmpC#	B1	O138	:H19	*iss, IpFA, mchF, iroN*
	45-W18	ESBL+AmpC#	A	09	:H30,H32	*iss*
	52-W18	ESBL+AmpC#	B1	0142	:H38	*iss, IpFA*
	55-W18	ESBL+AmpC#	A	O9	:H30	*iss*
Totara Valley (TL)	**1-S18**	ESBL+AmpC#	B1	-	:H2	*iss, IpFA*
	2-A18	ESBL+AmpC#	B1	O133	:H7	*iss, IpFA, cdtB*
	12-A18	ESBL+AmpC#	B1	O82	:H8,H40	*iss, IpFA, cdtB*
	54-S18	ESBL+AmpC#	B1	O152	:H2	*IpFA, astA*
	56-A18	ESBL+AmpC#	B1	-	:H39	*iss*
	87-A18	ESBL+AmpC#	B1	-	:H10	-

air=enteroaggregative immunoglobulin protein: *astA*=heat stable enterotoxin 1: *cdtb*=cytolethal distending toxin B *eiLA*=Salmonella HilA homolog: *gad*=glutamate decarboxylase: *iha*=adherence protein: *iss*=increased serum survival: *IpFA*=long polar fimbriae

Metadata of all 20 DSfSEC isolates were submitted to the NCBI Data benk and accorded accession numbers listed in Table 7.13

Table 7.13. NCBI accession number of DfSEC isolates

Isolate	Organism	NCBI accession number
CW28A18	*E. coli*	SAMN21857572
CW33S18	*E. coli*	SAMN21873234
CW49A18	*E. coli*	SAMN21873284
MRD21A18	*E. coli*	SAMN21873318
MRD22W18	*E. coli*	SAMN21873423
MRD24S17	*E. coli*	SAMN22130149
PF14A18	*E. coli*	SAMN22130150
PF15A18	*E. coli*	SAMN21894888
PF25S18	*E. coli*	SAMN22014245
PF30A18	*E. coli*	SAMN21904186
PF32A18	*E. coli*	SAMN21905091
PF45W18	*E. coli*	SAMN22251002
PF52W18	*E. coli*	SAMN22231228
PF55W18	*E. coli*	SAMN21923850
TL12A18	*E. coli*	SAMN21926700
TL1S18	*E. coli*	SAMN22216216
TL2A18	*E. coli*	SAMN21926618
TL54S18	*E. coli*	SAMN21976240
TL56S18	*E. coli*	SAMN22130148
TL87A18	*E. coli*	SAMN22131171

7.3 Discussion

According to the classification by Bush (2013), β-lactamase enzymes may be classified into A, B, C, and D groups. In this study, all 20 selected DfSEC were found to contain nucleotide sequences similar to the nucleotide sequence of either *bla*ACT-1, *bla*BIL-1, *bla*CMY-2, or *bla*MIR-1 gene variants that encoded for AmpC (group C) enzyme. The AmpC enzyme confers resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor-β-lactam combinations (Jacoby, 2009). The isolates PF32A18 and PF55W18, further carried the ESBL *bla*TEM-1 gene variant as confirmed by their genomic analysis using the Nullarbor and Geneious™Pro5.6.7 bioinformatics software. This genetic character had been confirmed by their phenotype disc diffusion antimicrobial susceptibility test against FOX30 and the MASTDISCS® (MAST™ Group Ltd, Liverpool, UK) phenotype screening assay (Chapter 6). This was in line with other studies that have undergone similar tests and assays to verify the existence of *bla* genes in *E. coli* strains from various niches (Durmaz, Bal, Gunaydin, Yula, & Percin, 2015; Ghaderi, Yaghoubi, Amirfakhrian, Hashemy, & Ghazvini, 2020; Shahcheraghi, Nasiri, & Noveiri, 2010; van den Bunt et al., 2019).

The chromosomally stemmed *bla*CMY-2, *bla*ACT-1, and *bla*MIR-1 genes (Reisbig & Hanson, 2004), originated from *Enterobacter cloacae* (Bradford, 2001) may be found on a transposon and transmitted to and from chromosomes and plasmids (Ku, Lee, Chuang, & Yu, 2018). All three genes may be located on a single transposon and transmitted together and are derived from older broad-spectrum β-lactamases such as *bla*TEM1&2 and *bla*SHV1 (Ku et al., 2018; Thomson, 2001). The *bla*ACT-1 gene of 381 amino acid length shared 94% homology with the 150 bp sequence of the *bla*MIR-1 gene with 86, 73, and 71% pairwise identities with AmpC β-lactamase genes from *E. cloacae* P99, *Citrobacter freundii* OS60, and *E. coli* K19, respectively (Barnaud et al., 1998). This was similar in this study where all 20 DfSEC isolates had nucleotide sequences similar to the nucleotide sequence of the ORF of either the *bla*ACT-1, *bla*BIL-1, *bla*CMY-2 or the *bla*MIR-1 gene variants and located at the same contig with similar coordinates and encoded for the production of the AmpC enzyme. The closeness in the relationship between the matched nucleotide sequences of the DfSEC

isolates and that of the nucleotide sequences of the ORF of the bla*ACT*-1, bla*BIL*-1, bla*CMY*-2, and the bla*MIR*-1 gene variants is also indicated by the phylogenetic tree created (Figure 7.7). This figure is an example for the rest of the DfSEC by using the isolates CW49A18 and CW28A18 with a gap penalty of 6 in the Geneious Pro bioinformatics software. Previous authors have used gap penalties between 1.5 (Das, Fearnside, Sarker, Forwood, & Raidal, 2017) and 12 (Pyo et al., 2014) in their studies.

The DfSEC isolates nucleotide sequence match with the ORF of the gene variants' similarity in the nucleotide sequence of these genes also indicated by the close resemblance of the phylogenetic tree created for the various DfSEC isolates using the ORFs of the *bla*ACT-1, *bla*BIL-1, *bla*CMY-2, and the *bla*MIR-1 gene variant ORF, compared to the comparatively different *tet*(34)1 gene variant with a gap penalty of 12 (Figures 7.2-7.6). The Geneious Pro bioinformatics software may treat nucleotide sequences that are not matched as null (Hwang, Han, Hong, & Han, 2017) instead of an A (adenosine), C (cytosine) G (guanine), or a T (thymine) because of the gap penalty (Zou et al., 2018). This may infer that the DfSEC isolates encode nucleotide sequence match for one of these gene variants only and not of multiple gene variants. Since the *bla*CMY-2 gene variant is the is abundant AMR gene in New Zealand (Jean, Hsueh, & Group, 2016; Karkaba, Hill, Benschop, Pleydell, & Grinberg, 2019) compared to the prevalence of the other three (*bla*ACT-1, *bla*BIL-1 and *bla*MIR-1), it may be speculated that in this study, the DFSEC isolates encode for the *bla*CMY-2 nucleotide sequence rather than any of the other three. In this study, the Geneious Pro bioinformatics software may not have identified mutations, deletions (INDELs), single nucleotide polymorphisms (SNPs), and variable number tandem repeats (VNTRs).

According to Poirel et al.(2018), the prevalence of various *bla* genes and other ARGs depends on the geographical location, the species from which the *E. coli* were isolated, and the pathological condition in question. This may explain the findings in this study as the *E. coli* isolates were sourced from bovine dairy farm soils. Other studies that have sourced *E. coli* from particular niches have shown that *E. coli* of a particular phylogenetic group, serovar, or a virulence determinant dominate that particular niche. Studies from France on 1427 *Klebsiella pneumoniae* isolates from bovine mastitis (Dahmen, Métayer, Gay, Madec, & Haenni, 2013), Spain on environmental, human and meat

sourced *E. coli* (Ojer-Usoz, González, & Vitas, 2017), Canada on *E. coli* from humans (Mulvey et al., 2005), South Korea on *E. coli* from beef cattle and the Czech Republic on *E. coli* from manure and milk (Kyselková, Jirout, Vrchotová, Schmitt, & Elhottová, 2015; Kyselková, Kotrbová, et al., 2015) have confirmed this phenomenon.

The *bla*DHA-1 may have originated from *Morganella morganii* as the AmpR and AmpC regions of the gene has a 98% intracistronic match with the genomic DNA of the organism and spread to other bacteria including members of the Enterobacteriaceae family as plasmid borne inducible gene (Barnaud et al., 1998; Verdet, Arlet, Barnaud, Lagrange, & Philippon, 2000). The *bla*DHA-1 has close similarities to the nucleotide sequences of the *bla*ACT-1, *bla*CMY-2, and *bla*BIL-1 gene variants at 98% and protein sequence similarity of between 53-58% according to a study by (Barnaud et al., 1998). In this study, the genomic nucleotide sequence of the DfSEC isolates had a sequence query coverage of > 88% and a pairwise identity of > 62% on the same contig as those of *bla*ACT-1, *bla*CMY-2, and *bla*BIL-1 gene variants and encoded for the production of AmpC protein (enzyme) and may also explain their phenotype resistances especially, to FOX30 for the two isolates CW49A19 and PF30A18 (Table7.6). The hydrolytic activity of the AmpC enzyme is similar irrespective of their origin (Reis et al., 2020) and this may explain the reduced resistance to the cephalosporin antimicrobial FOX30 (Jacoby, 2009; Pitout, 2012b) (Table A1.1).

Generally, the *bla* genes in Enterobacteriaceae are not always found in all parts of the globe at the time of their initial discovery (Khoshbakht, Shahed, & Aski, 2020; Lübbert et al., 2015). Presently the closely related large family of β-lactam hydrolysing enzymes of *bla*CTX-M, *bla*SHV*,* and *bla*TEM gene variants are found worldwide (Ojdana et al., 2014; Toombs-Ruane et al., 2017). These phenomena may indicate a gradual spread from a common source to other parts of the world as was the case for the New Delhi-Metallo-β-lactamase gene variant (*bla*NDM-1) transmission from Indian to New Zealand by human migration (López-Cerero & Almirante, 2014; Shahid et al., 2014; Williamson et al., 2012). The *bla*TEM gene variants together with its closely associated genes of *bla*CTX-M and *bla*SHV variants were the first of the β-lactamases to be described (Poirel et al., 2018; Toombs-Ruane et al., 2017). These resistance genes have numerous variants, with newer ones continuously being

discovered in different geographical locations and niches and have the highest prevalence of resistance to ESBLs in Enterobacteriaceae, especially *E. coli* (Liakopoulos, Mevius, & Ceccarelli, 2016; Michael et al., 2015). In New Zealand, the most prevalent *bla*ESBL, mostly from human sources, is the CTX-M type (Freeman et al., 2012; Heffernan, Dyet, Woodhouse, & Williamson, 2014). Similarly, among domestic cats and dogs, the *bla*CTX-M type is the next most common, followed by a *bla*CMY-2 variant (Karkaba et al., 2019). This is comparable to data from other parts of the world. A study of the prevalence of ESBL producing *E. coli* on a Bavarian dairy and beef cattle farm (Schmid et al., 2013) found the most prevalent gene variant to be the *bla*CTX-M-1 gene variants at 96%, 23% for the *bla*CMY-2 gene, and only 2.5% for AmpC type. A Chinese study of ESBL resistant *E. coli* isolates from bovine mastitis found 78%, 56%, and 16% of the isolates either in singlet or combination to be *bla*CTX-M, *bla*TEM, and *bla*SHV genes variants respectively (Yang et al., 2018).

In this study, the *mdf*A gene was common to all the DfSEC isolates investigated, which is in line with the suggestion that the *mdf*A gene is intrinsic to *E. coli* strains (Heng et al., 2015; Sigal et al., 2005) and common to most bacteria (Lewinson et al., 2003). According to Levy (2002), the *mdf*A gene is activated by environmental signals or by mutation of a regular gene for expression. The *E. coli mdf*A gene is of 410 amino acid residues arranged in 12 transmembrane helices with their N and C terminals embedded in the cytoplasm (Adler & Bibi, 2002). This amino acid sequence encodes for a member of the major facilitator superfamily of proteins which confers resistance to a wide variety of compounds toxic to bacteria, including antimicrobials of different structures and mechanisms of action (Edgar & Bibi, 1997; Heng et al., 2015; Nagarathinam et al., 2017). This was biologically demonstrated in a study by exposing mutant and wild-type strains of *E. coli* to high concentrations of biologically toxic ethidium bromide and chloramphenicol (Sigal, Molshanski-Mor, & Bibi, 2006). The role of the *mdf*A gene in reducing the minimum inhibition concentration of fluoroquinolones by the efflux pump was also demonstrated in clinical *E. coli* isolates by Swick, Morgan-Linnell, Carlson, and Zechiedrich (2011). This may explain the reduced susceptibility of these 20 DfSEC isolates in this study to cefoxitin, together with tetracycline resistance in four others.

In this study, all 20 DfSEC encoded similar nucleotide sequences to the ORF of the *tet*(34)1 resistant gene to tetracycline(67% coverage and 74% pairwise identity), but only four of the isolates were phenotypically resistant to it. In a South Korean study of 146 *E. coli* isolates from beef cattle that were resistant to tetracycline, none of the isolates carried the *tet*(34)1 gene variant but rather *tet*(A), *tet*(B), and *tet*(C) at 46%, 45%, and 7%, respectively (Shin, Shin, Jung, Belaynehe, & Yoo, 2015). Also, in a Czech Republic study that looked at the occurrence of *tet* genes in the faeces, manure, and milk of cows of various ages from conventional dairy farms, after the farm's routine use of intrauterine tetracycline suppository, the authors found all the various *tet* genes variants to be the age of cow and manure dependant. The authors also found that *tet* (W), *tet*(O), and *tet*(Q) predominated in all samples, and *tet*(A), *tet*(M), *tet*(Y), and *tet*(X) were less so. No *tet*(34)1 was found in this study apart from samples taken from aged manure at 2 m deep (Kyselková, Jirout, et al., 2015). These findings were similar to the USA study (Yang et al., 2020). In this study, the soil samples from which the *E. coli* strains were isolated, were taken from the surface to 30 cm of soil depth with conditions which may be equated to that depth of the aged manure and may explain the findings of *tet*(34)1 gene variant in 18 of the 20 DfSEC isolates investigated by the Nullarbor bioinformatics software (Table 7.2).

In this study, the nucleotide match between the DfSEC isolates and the ORF of the *tet*(34)1 gene variant encoded for the production of the metabolic protein xanthine-guanine phosphoribosyltransferase, an intrinsic *E. coli* enzyme (Krenitsky, Neil, & Miller, 1970). This enzyme is involved in the metabolism of purine, xanthine, and guanine amino acids in the purine salvage pathway of *E. coli* (Vos, Parry, Burns, de Jersey, & Martin, 1998) for the synthesis of DNA, RNA, and the generation of ATP during biogenesis (de Souza Dantas, Ramos dos Santos, Guimarães Pereira, & Medrano, 2008). This is constituent with the findings in this study as, although all 20 DfSEC isolates carried similar nucleotide sequence that matched the nucleotide sequence of the ORF of the *tet*(34)1 gene at 67.3% coverage and 75.1% pairwise identity, only 4/20 (PF15A18, PF25S18, MRD21A18, and MRD22A18) of the isolates were phenotypically resistant to Te(30) (Table A1.1). Organisms may genetically encode a resistant gene but not express the resistance phenotypically (Mbelle et al., 2019). The resistance showed by these four isolates may be due to their ability to hyper-express the

efflux pump mechanism to extrude tetracycline from the cytoplasm by active transport, (Blair, Richmond, & Piddock, 2014; Roberts, 2005) or by the use of ribosomal protection proteins, and modification of the drug (Roberts, 2005; Thaker et al., 2010).

In this study, apart from all the 20 DfSEC submitted for WGS demonstrating the potential to harbour multiple resistance genes to different antimicrobial groups, and phenotypically being resistant to the cefoxitin (FOX30), 3.7% of the 814 DfSEC isolates from both dairy farming systems showed varying susceptibility/resistance to other groups of antimicrobials. Such a result was confirmed by other studies on *E. coli* from different niches (Sunde & Norström, 2006; Wu et al., 2012).

In this study, there were differences in the output of the Nullarbor and Geneious™ Pro 5.6.7 bioinformatics software. These differences could be attributed to the parameters set to detect the resistant determinants used in the two analyses. For the genotype-phenotype determinant in the Geneious™ Pro 5.6.7, the homologous nucleotide sequence between the ORF of the gene variant under consideration and the genome assembly of the DfSEC at > 80% and pairwise identity at > 70% was used. The matching nucleotide sequence was NCBI BLAST queried for the encoding protein (enzyme) as the genotype-phenotype AMR determinant. For the Nullarbor analysis, however, the genome sequences of the DfSEC isolates were put through the ResFinder4.0 software (Bortolaia et al., 2020). The ResFinder4.0 software was set up using four different databases including translations of genotype into phenotype by a panel of experts who reviewed AMR determinants from applicable literature (Bortolaia et al., 2020) with the query cover at 2/5 length of the resistant gene and pairwise identity at 98%. This explains the matching outputs of the Geneious™ Pro 5.6.7 and Nullarbor analysis regarding the *bla*TEM-1 resistant gene (Geneious™ Pro 5.6.7 pairwise identity was at 99.9%) but not with *bla*ACT-1, *bla*BIL-1, *bla*CMY-2 and *bla*DHA and *bla*MIR-1 resistant gene variants whose pairwise identity were set at > 70%.

In Enterobacteriaceae such as *E. coli,* resistance to an extended-spectrum β-lactam antimicrobial is achieved in three major ways: excretion of the corresponding hydrolysing enzymes to the particular β-lactam antimicrobial (Noval, Banoub, Claeys, & Heil, 2020; Pfeifer & Eller, 2012), the use of cell wall transpeptidases that antimicrobials do not bind to, or by active removal of the particular

antimicrobial from the cell (Wilke, Lovering, & Strynadka, 2005). The biochemical structure of enzymes involved in the process of crosslinking the peptidoglycan molecule to an antimicrobial structure and the change in the structure of a target site of membrane-bound serine transpeptidases play a crucial role in the bacteria's ability to resist the effects of an antimicrobial (Lobanovska & Pilla, 2017). Similarly, modification of the chemical structure of most antimicrobials, such as the side-chains of the β-lactam ring, results in new activities of the drug and mostly an expansion of the spectrum of the group of antimicrobials (Cooper, Hatfield, & Spry, 1973; Page, 2012; Yamamoto et al., 1982). Thus, the chemical structure of the proteins or family of proteins involved in the three-step/three-site biogenesis of the bacteria cell wall would play vital roles in the susceptibility/resistance of a bacteria to an antimicrobial (Kong, Schneper, & Mathee, 2010). In this study, the DfSEC isolates that showed resistance to antimicrobials all carried nucleotide sequences that encoded for proteins or family of proteins which would help the cell avoid destruction by one or the other mechanism described above.

Globally, ESBL-producing *E. coli*, as well as *E. coli* resistant to third and fourth cephalosporins like cefoxitin, are widespread (Padmini, Ajilda, Sivakumar, & Selvakumar, 2017). A 2009-2012 data from a study initiated in 2002 in the Asia-Pacific region to study the trend of AMR in 1762 Enterobacteriaceae isolates of which 56.3% were *E. coli* collected from 38 different hospitals from 10 countries in the region, including three hospitals in New Zealand, indicated that 28%, 50.3% and 74% of the total number of Enterobacteriaceae isolates were susceptible to extended-spectrum beta-lactam antimicrobials, third and fourth generation cephalosporins, respectively (Lu et al., 2012). A similar study of 903 *E. coli* isolates from women aged between 18-65 in five European countries (Austria, Greece, Portugal, Sweden, and the UK) with uncomplicated lower urinary tract infection (UTI) also found 120 (13.3%) to be resistant against four or more (multi-resistant) of the antimicrobial agents used in the study (Kahlmeter & Poulsen, 2012). The commonest resistant genes in the European study (Kahlmeter & Poulsen, 2012) were of the CTX-M and AmpC variants. Similarly, an Asia-Pacific region study (Jean, Hsueh, et al., 2016) found the *bla*CTX-M and *bla*CMY-2 resistant genes to be most common. In this study, of the total 814 DfSEC studied, 2.45% (20/814) were found

to harbour multi-resistant genes of seven gene variants, and close similarity to the nucleotide sequence of the ORF of the *bla*ACT-1, blCMY-2, *bla*MIR-1 genes were common to all 20 multi-resistant isolates according to nucleotide sequences analysis by the Geneious™ Pro at 95% coverage and 73% pairwise identity. A Southern Pennsylvania, USA study on the widespread nature of antimicrobial resistance genes from lactating cows' faeces and soil around the cattle barns was examined. It was found that the most abundant genes were the multidrug-resistant genes (44.7%), vancomycin-resistant genes (12.5%), tetracycline at 10.5%, bacitracin (10.4%) and β-lactam resistance genes were at 7.1%, MLS efflux pump (6.9%) (Pitta et al., 2016). The major ESBL resistant genes include *bla*TEM, *bla*SHV, and *bla*CTX-M and are found all over the world (Bora et al., 2014; Coque, Baquero, & Cantón, 2008), while others such as *bla*OXA (Evans & Amyes, 2014) may be relative, and with plasmids, play a crucial role in their transmission and spread (Benz et al., 2021).

A New Zealand study that looked at the relatedness of *Streptococcus uberis* from the mammary glands of cows with clinical and subclinical mastitis from different dairy herds from different districts using the genomic tool of pulsed-field gel electrophoresis indicated dissimilarities between isolates from different herds but similarities between isolates from different quarters of the same cow (Douglas, Fenwick, Pfeiffer, Williamson, & Holmes, 2000). A similar study on Shiga toxin-producing *E. coli* (STEC) from 102 farms in different regions of New Zealand using PCR/MALDI-TOF genomic tools indicated the possibility of *E. coli* spread within a farm but not between farms (Browne et al., 2018). In this study, the minimum spanning tree generated from the Nullarbor analysis of the 20 DfSEC isolates indicated some level of relatedness between two isolates from different farms (TL1S18 and CW33S18) (Figure 7.8). The use of wgMLST as a genomic tool for the relatedness study of bacteria species is more sensitive than the use of pulsed-field gel electrophoresis (David et al., 2013; Schouls et al., 2009). In the New Zealand dairy farming industry, it is common and traditional for farms to share contractors such as milk buying companies, feed, and fertiliser suppliers and to tread common walk-ways (Hidano, Gates, & Enticott, 2019; Kerr & Layton, 1983), especially on moving days (https://www.dairynz.co.nz/business/moving-day/managing-contractors/). This phenomenon, as well as running waterways (McWilliam & Balzarova, 2017) and wild animals including birds, may

cause the spread of microbes between farms located in the same catchment or area. This may explain findings in this study where some *E. coli* isolates from a particular farm showed close relatedness to another isolate from a different farm.

In New Zealand, 2016 data indicated 8.9 cases per 10 000 people of STEC with living near cattle or contact with cattle and their manure/faeces as the principal sources of infection, but not from food (Browne et al., 2018; Jaros et al., 2016). The STEC O157:H7 is most commonly known globally, for the severe morbidity and mortality it causes (Akindolire, 2019). According to Coombes et al. (2008), there are over 200 highly virulent non-O157 STEC serotypes that can be found worldwide. A 2008 study (Leotta et al., 2008) of STEC-types found in Argentina, New Zealand and Australia indicated that in Argentina and New Zealand most haemolytic uraemia syndromes were caused by the STEC O157:H7 types but less so in Australia. In that study, the 76 *E. coli* strains could be divided into 10 different clusters, 46 different patterns with 36/76 strains showing unique patterns, indicating the wide variability and close relatedness that these organisms share, similar to findings in this study. In New Zealand (Jaros et al., 2013) as well other parts of the world (Jaros et al., 2014; Leotta et al., 2008) cattle are considered to be the main reservoirs of STEC and is a frequent source of infection in human outbreaks. A New Zealand study of 91 STEC strains from rectal swabs of cattle and 48 strains from sheep showed the majority of them to be of H8 or H10 flagellum subtype (Cookson et al., 2010). Similarly, in this study, 2/20 DfSEC isolates had the H8 flagellum and 1/20 was of the H10 type. In another Australia/New Zealand study involving data from 182 human patients and isolated 179 cephalosporin-resistant *E. coli* strains had 39% of isolates were of the H30 subtype (Rogers et al., 2015), while in this study 2/20 (10%) of isolates were of the H30 subtype.

The data from this study did not include any of the more prevalent pathogenic subtypes of O157, O26, O111, or O145 (Cookson et al., 2010) subtypes, the presence of H8 and H30 subtypes may be of concern to human health and caution in dealing with *E. coli* isolates from the soil of dairy farms may need to be advocated for. This is because, in the present study, toxins and the toxicity level of the DfSEC isolates were not looked at. Such a test could be done in the future to shed more light on the

variability of *E. coli* isolates and the potential danger that commensal to cattle but virulent to humans (Jaros et al., 2013) *E. coli* may pose to public health risks.

Chapter 8

Conclusions of the study

Until the onset of the Covid-19 pandemic, the topmost priority of not only the WHO, but the FAO, and centres for disease controls around the world, was antimicrobial resistance (AMR) development, prevalence, and spread (Silva et al., 2021; World Health Organization, 2020). AMR is expected to overwhelm health facilities, derail a century's worth of medical progress, and cause more deaths than cancer by the year 2050 (Bhatia, 2018). Scientists around the globe have questioned how best to control AMR development, prevalence and have put efforts into discovering new potent pharmaceuticals (Tacconelli et al., 2018). Arguably, the cost in financial terms, quality of life, and other societal losses caused by AMR can never be accurately or near accurately assessed (World Health Organization, 2014). The surveillance which governments are encouraged to carry on the development, prevalence, and spread of AMR to help address the situation, would be enhanced by a baseline study of the current situation. This could be followed by the frequent comparison of industries that use chemicals including agrochemicals and antimicrobials with industries that use these chemicals sparingly or not at all.

The design of this study was aimed at providing valuable information on the current AMR situation in the Geraldine area of South Canterbury, New Zealand. The phenotype and genotype AMR status of the widely accepted indicator organism of AMR, *E. coli*, in the soils of the fast-growing dairy industry was looked at by comparing isolates from two organic dairy farms to their counterparts from two conventional farms in the district.

In New Zealand, as well as most developed countries the use of antimicrobials is the key component in the control of the most economically important disease of mastitis and other infectious diseases in the dairy industry (McDougall, 2001, 2002; McDougall & Compton, 2002; McDougall et al., 2017). Antimicrobials are used annually in New Zealand conventional dairy farms as opposed to non-usage in organic dairy farms for the prevention and control of mastitis and end up in the soils of the fields

(Tasho & Cho, 2016). Secondly, dairy farming in New Zealand is pastoral based and the need to control weeds on the pastures and in fodder feed is critical and herbicides including salts of glyphosate and 2, 4-D Ethylhexyl ester is commonly used (Ghanizadeh & Harrington, 2019; Manktelow et al., 2005) on conventional dairy farms but not on organic dairy farms. These agrochemicals in combination with antimicrobial usage contribute to the development of antimicrobial resistance in the soils (Kurenbach, Hill, Godsoe, van Hamelsveld, & Heinemann, 2018; Van Bruggen et al., 2018). The phenotype and genotype AMR status of dairy farm soil *E. coli* (DfSEC) isolates from conventional dairy farms with regular use of antimicrobials and other agrochemicals were compared to that of their counterparts from the organic dairy farms with limited/non-usage of antimicrobials and other agrochemicals. The soil harbours the widest range of strains and the highest amount of any particular bacteria species (Lauber, Hamady, Knight, & Fierer, 2009; Yergeau, Newsham, Pearce, & Kowalchuk, 2007). Since microorganisms are the source of most antibiotics, it follows that antibiotics can be found in all soils containing bacteria on the planet irrespective of human intervention (Wellington et al., 2013). The soil acts as a depot for industrial, agricultural, and wastes of all kinds (Alloway, 1996; Gorovtsov et al., 2019). It may be expected that any soil with a history of human industrial, domestic, and/or agricultural wastes would contain antimicrobials and other chemicals that may not only accelerate the development of AMR in the soil's bacteria population (Baquero & Blázquez, 1997; Davies & Davies, 2010; Michael et al., 2014) but would also contain a larger variety of antimicrobial-resistant genes (ARGs) and their variants compared to areas without human intervention (Qi et al., 2019).

It is possible to deduce from the findings in this study that, presently, the conventional dairy farming system in the Geraldine area may not have impacted the development of AMR in DfSEC isolates to any significant extent compared to their organic counterparts but there is a tendency for the *E. coli* found in the conventional dairy farm soils to possess more ARGs. Similarly, in a Swiss study, the antimicrobial resistance status of different species of bacteria to a selected group of antimicrobials did not find any significant differences between the two farming systems regarding their influence on the antimicrobial resistance of the majority of bacteria species studied (Roesch et al., 2006).

However, a study in the USA of *Staphylococcus aureus* from the milk of conventional dairy farm cows compared to their counterparts from organic dairy farms to selected antimicrobials (Tikofsky, Barlow, Santisteban, & Schukken, 2003) found the conventional dairy farming system to significantly influence the antimicrobial resistance status of *Staphylococcus aureus* in their milk.

In this study, among the 20 isolates selected for genomic analysis, those with the highest number of ARGs were from the conventional Peel Forest dairy farm soil, with two of them having up to six ARGs. This would imply that at any one time, there would be more resistant gene variants in circulation in the soils of the conventional dairy farms compared to their organic counterparts, in the Geraldine district. The second finding from this study was that some of the highly prevalent antimicrobial resistant genes recorded from other parts of the world and New Zealand, *bla*CTX-M and *bla*SHV were not found in the Geraldine catchment of South Canterbury dairy farm soils. The presence of *bla*CMY-2 variant, second in abundance in New Zealand (Jean, Hsueh, et al., 2016; Karkaba et al., 2019) may be concluded in a future study. The non-prevalence of the most commonly found ARGs of *bla*CTX-M and *bla*SHV and the low prevalence of AMR DfSEC in the dairy farming systems of the Geraldine district may be related to a) its comparatively remote location. Geraldine is a rural community, distant away from the nearest industrial and heavily populated cities. The nearest industrialised city to the Geraldine district of South Canterbury is Christchurch, 140 km away. b) The waterways including rivers, streams, and creeks in the Geraldine district are sourced from melted snow from atop the mountain ranges of the Southern Alps located to the west of the district. This implies there are very little if any contaminants of heavy metals and other chemicals from industries and human settlements to compound AMR development. c) The pastoral (paddock-based) dairy farming and the mild climatic conditions in the Geraldine area means the stock is held on a wide area of land all year round compared to other OECD countries (Barry, 1974; Flysjö, Henriksson, Cederberg, Ledgard, & Englund, 2011) and antimicrobials (Jechalke, Heuer, Siemens, Amelung, & Smalla, 2014) and other agrochemicals (Groot & van't Hooft, 2016; Sparks & Lorsbach, 2017) are not deposited in a defined area to enhance AMR development. A review of the role that intense deposition and accumulation of

agrochemicals especially glyphosate plays in the development of AMR indicated a strong correlation between glyphosate use and AMR development in the environment including soils (Van Bruggen et al., 2018). The situation in a conventional dairy farm in Geraldine may be equated to an organic farm in some other OECD countries in terms of the intensity of antimicrobials (Jechalke et al., 2014; Levy & Marshall, 2004), agrochemicals (Kurenbach et al., 2018; Kurenbach et al., 2015; Mulder et al., 2018), and industrialised waste (Graham, Knapp, Christensen, McCluskey, & Dolfing, 2016; Graham et al., 2011) deposition, which is widely accepted to contribute significantly to AMR development.

It is traditionally acceptable in the New Zealand dairy farming industry for stock to migrate once a year from different areas of a district to the other (Hidano et al., 2019), share contractors, and even waterways (McWilliam & Balzarova, 2017). The results of this study indicated the interrelatedness of some of the isolates, two of which were from different farms. This shed light on how some of these practices may aid the spread of ARGs in the district. This may affect most undertakings on the land and the possible spread of ARGs to humans and wildlife. In this study, it was established that the more pathogenic and virulent phylogenetic groups A (Blum & Leitner, 2013; Picard et al., 1999) and E (Tomazi et al., 2018) may be found in the dairy farm soil, albeit, in lesser quantity. This would mostly be caused by the culture of disposing of milk from cows with mastitis into the farm's sewage system and later used for irrigation of the fields. The feeding of such milk to calves (Abb-Schwedler et al., 2014) could also be a factor as intestinal microbes are easily circulated between the livestock and the farm soil on a dairy farm and transfer of genetic material, either vertically or horizontally amongst strains of the same species and between species is common (Benz et al., 2021). Further, humans, as well as their pets may share microbes with the livestock on a farm. As it is not uncommon for cows with subclinical mastitis to shed microbes in their milk that would be consumed by humans, cats, and dogs on the farm. In this study, AMR gene variants common to all 20 DfSEC as detected by the genomic analysis of sequenced *E. coli* indicated the presence of intrinsic to Enterobacteriaceae family, efflux pump, and the AmpC type β-lactamase was expected of DfSEC (Cox & Wright, 2013; Ma et al., 1995; Weston, Sharma, Ricci, & Piddock, 2018). In this study, it can be argued that the two isolates out of the 20 isolates that have multi-antimicrobial resistance, belonged to the phylogenetic

group A and carried the multi-resistant *fim*H30 allele (Krekeler et al., 2012; Matsumura et al., 2015; Roer et al., 2017) may be of bovine mammary or uterine tissue origin and may have caused mastitis or metritis, respectively, in the cow of origin. The pathogenesis of mastitis is such that subclinical mastitis caused by *E. coli* may go undetected for some time (Ruegg, 2017). Milk from such cows if consumed by humans without pasteurisation by farm gate sales may cause infection or carriage of ARGs from cows to humans (Watts, 2015). Further, secretions from cows with metritis may contain a good amount of pathogenic *E. coli* that may be deposited onto the fields causing such strains of *E. coli* to get established in the soil (Burfeind et al., 2014).

In this study, the exclusion of any antimicrobial in the isolation media means the method developed recovered a diverse range of dairy farm soil *E. coli* isolates suitable for antimicrobial resistance studies. Using a method of isolating environmental bacteria without antimicrobial(s) reduces bias to the select strains in an antimicrobial susceptibility/resistant study as the researcher would be naïve to the antimicrobial status of the selected strains. In this study, the diversity in the phenotype and genotype characteristics of *E. coli* strains obtained, were indicted by the wide phylogenetic grouping, phenotype, and genotype antimicrobial characteristics. This method may easily be applied to the isolation of diverse species of bacteria from various environmental niches in a cost-effective manner. The method does not require sophisticated equipment and high-cost reagents but results in high sensitivity and specificity in isolation. The isolation protocol can be adopted by the simplest of microbiological laboratories for high thorough-put results.

The choice of sampling sites for this study that were geographically closely located eliminated any bias that may have resulted from climate/weather, soil-type, vegetable, and agricultural practices which would influence the characteristics of the *E. coli* strains for the study. As it is well established that *E. coli* strains from different niches are affected by the conditions prevailing in these niches. More so, the collection of samples from the paddock gate to the nearest watering trough, that was adopted in this study, may have increased the chance of isolating diverse *E. coli* strains that may have had interaction with the majority of stock held on the paddock. This is because it is habitual for cows

to walk straight to the watering troughs upon returning to the paddock after milking sessions. The inclusion of ten paddocks per farm would also have helped.

In a USA study, a correlation between the human impact of antimicrobial deposition into dairy farm soil and AMR development was made when an initial study and a study after 14 years of antimicrobial deposition was conducted (Yang et al., 2020). This is the first study of this kind in the Geraldine district and similar future studies may indicate a trend as a result of the two different farming systems. Further, future studies that would include samples taken from the same farms as this study, and other dairy farms from different regions of the country, especially from heavily populated towns and cities with heavy industries would help to establish a solid relationship between human activities and the development of AMR, its prevalence and mode of spread as has been hypothesised by this study. Such a study could also be extended to other agricultural domains such as crop and vegetable farms that routinely use agrochemicals. Further, continuous sampling and coverage of broader geographical areas in New Zealand would help establish an AMR surveillance system.

Another value of the design of the study was the choice of the widely accepted indicator of AMR development, *E. coli*. *E. coli* is ubiquitous in most environments. The diversity in its strains, characteristics, and quick generation time of approximately 20 min eases its use in studies regarding changes in its phenotype and genotype. The information this study provided would be most valuable in the control and monitoring of antimicrobial resistance in the dairy industry, as it is possible to control acquired resistances, not intrinsic ones. Such findings would alert the relevant bodies such as veterinary groups, the Ministry for Primary Industries, and other stakeholders of the industry to not only the presence of resistant elements but their prevalence and mode of spread.

This was a baseline study and similar studies in the future would showcase a trend of AMR development by the dairy farming industry. Such a study has been conducted in various regions around the globe and has led to the initiation of mitigation programs to control the emergence and spread of antimicrobial resistance (De Kraker et al., 2013; Jean, Coombs, et al., 2016). The different species of bacteria such as *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Staphylococcus*

aureus commonly associated with bovine mastitis in New Zealand respond differently to the antimicrobial groups (McDougall, Hussein, & Petrovski, 2014; Petrovski, Laven, & Lopez-Villalobos, 2011). Future studies that would compare the phenotype and genotype profiles of these different bacteria species may shed more light on the role the use of antimicrobials in conventional dairy farms may play in the development of antimicrobial resistance. Further, comparing bacteria isolated from the mammary glands (McDougall et al., 2014), respiratory (Bassel, Tabatabaei, & Caswell, 2020), or digestive (Moriarty, Sinton, Mackenzie, Karki, & Wood, 2008) system of cows to those isolated from the bovine external environment. Such a study may also highlight the effect that physical features like the differences in temperature, pH, and UV exposure have on the antimicrobial susceptibility of different species of bacteria isolated from the environment and different mammalian physiological systems.

In this study, the genomic analysis of the 20 DfSEC isolates, which showed reduced susceptibility to especially the broad-spectrum second-generation cefoxitin of cephamycins group and their phylogenetic grouping enabled the evaluation of the possible source/cause of AMR *E. coli* and antimicrobial resistance genes (ARGs) on dairy farms. This study may be extended to include other animal husbandry systems, vegetable, and crop farms. Secondly, a Swiss study (Roesch et al., 2006) suggested that the difference between the influence of the two dairy farming systems may be bacteria species-oriented and future studies could be designed to look at other bacteria species such as *Streptococcus* and *Staphylococcus* spp. important in the dairy industry apart from *E. coli*.

Chapter 9

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Appendix AD

Antimicrobial profile of dairy farm *E. coli* isolates

Table A1.1 Antimicrobial susceptibility/resistance profile of DfSEC by the disc diffusion method

Disc diffusion antimicrobial susceptibility testing

Clearwaters organic dairy farm. Spring 2017.

S.No	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening test	Phylogenetic group
1	CW-16-S17	R	S	I	S	S	S	R	S	nil	B1
2	CW-35-S17	S	S	S	S	S	S	S	S	nil	B1
3	CW-46-S17	S	S	I	S	S	S	R	S	nil	B1
4	CW-60-S17	R	S	I	S	S	S	S	S	nil	B1
5	CW-1-S17	R	S	S	S	S	S	S	S	nil	B1

Disc diffusion antimicrobial susceptibility testing Clearwaters organic dairy farm. Spring 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening test	Phylogenetic group
1	CW-19-S18	S	S	S	S	S	S	S	S	nil	C
2	**CW-33-S18**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**E**
3	CW-2-S18	S	S	S	S	S	S	S	S	nil	B1
4	CW-3-S18	S	S	S	S	S	S	S	S	nil	B1
5	CW-24-S18	S	S	S	S	S	S	S	S	nil	B1
6	CW-25-S18	S	S	S	S	S	S	S	S	nil	E
7	CW-20-S18	S	S	S	S	S	S	S	S	nil	E
8	CW-30-S18	S	S	S	S	S	S	S	S	nil	E
9	CW-31-S18	S	S	S	S	S	S	S	S	nil	E
10	CW-32-S18	S	S	S	S	S	S	S	S	nil	E
11	CW-8-S18	S	S	S	S	S	S	S	S	nil	E

12	CW-6-S18	S	R	S	S	S	S	S	S	nil	B1
13	CW-5-S18	S	S	S	S	S	S	S	S	nil	B1
14	CW-27-S18	S	S	S	S	S	S	S	S	nil	E
15	CW-17-S18	S	S	S	S	S	S	S	S	nil	B1
16	CW-34-S18	S	S	S	S	S	S	S	S	nil	E
17	CW-29-S18	S	S	S	S	S	S	S	S	nil	B1
18	CW-18-S18	S	S	S	S	S	S	S	S	nil	E
19	CW-11-S18	S	S	S	S	S	S	S	S	nil	B1
20	CW-12-S18	S	S	S	S	S	S	S	S	nil	B1
21	CW-9-S18	S	S	S	S	S	S	S	S	nil	B1
22	CW-35-S18	S	S	S	S	S	S	S	S	nil	E
23	CW-13-S18	S	S	S	S	S	S	S	S	nil	B1
24	CW-15-S18	S	S	S	S	S	S	S	S	nil	C
25	CW-14-S18	S	S	S	S	S	S	S	S	nil	C

26	CW-7-S18	S	S	S	S	S	S	S	S	nil	C
27	CW-10-S18	S	S	S	S	S	S	S	S	nil	B1
28	CW-40-S18	S	S	S	S	S	S	S	S	nil	B1
29	CW-44-S18	S	S	S	S	S	S	S	S	nil	E
30	CW-36-S18	S	S	S	S	S	S	S	S	nil	B1
31	CW-38-S18	S	S	S	S	S	S	S	S	nil	B1
32	CW-43-S18	S	S	S	S	S	S	S	S	nil	B1
33	CW-46-S18	S	S	S	S	S	S	S	S	nil	B1
34	CW-45-S18	S	S	S	S	S	S	S	S	nil	B1
35	CW-22-S18	S	S	S	S	S	S	S	S	nil	B1
36	CW-28-S18	S	S	S	S	S	S	S	S	nil	B1
37	CW-23-S18	S	S	S	S	S	S	S	S	nil	B1
38	CW-39-S18	S	S	S	S	S	S	S	S	nil	D
39	CW-42-S18	S	S	S	S	S	S	S	S	nil	B1

40	CW-41-S18	S	S	S	S	S	S	S	S	nil	B1
41	CW-16-S18	S	S	S	S	S	S	S	S	nil	C
42	CW-26-S18	S	S	S	S	S	S	S	S	nil	E
43	CW-51-S18	S	S	I	I	S	S	I	S	nil	B1
44	CW-47-S18	S	S	R	S	S	S	S	S	nil	B1
45	CW-49-S18	S	S	S	S	S	S	S	S	nil	B1
46	CW-50-S18	S	S	S	S	S	S	S	S	nil	B1
47	CW-46-S18	S	S	S	S	S	S	S	S	nil	B1

Isolate in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing. Clearwaters organic farm. Autumn 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	CW-16-A18	R	S	S	S	S	S	S	S	nil	E
2	CW-15-A18	S	S	S	S	S	S	S	S	nil	Unknown
3	CW-1-A18	S	S	S	S	S	S	S	S	nil	E
4	CW-2-A18	S	S	S	S	S	S	S	S	nil	B1
5	CW-10-A18	S	S	S	S	S	S	S	S	nil	B1
6	CW-14-A18	S	S	S	S	S	S	S	S	nil	E
7	CW-3-A18	S	S	S	S	S	S	S	S	nil	B1
8	CW-9-A18	S	S	S	S	S	S	S	S	nil	B1
9	CW-31-A18	S	S	S	S	S	S	S	S	nil	A
10	CW-43-A18	S	S	S	S	S	S	S	S	nil	B1
11	CW-6-A18	S	S	S	S	S	S	S	S	nil	C

12	CW-8-A18	S	S	S	S	S	S	S	S	nil	E
13	CW-39-A18	S	S	S	S	S	S	S	S	nil	C
14	CW-44-A18	S	S	S	S	S	S	S	S	nil	E
15	CW-36-A18	S	S	S	S	S	S	S	S	nil	E
16	CW-20-A18	S	S	S	S	S	S	S	S	nil	B1
17	CW-23-A18	S	S	S	S	S	S	S	S	nil	E
18	CW-48-A18	S	S	S	S	S	S	S	S	nil	B1
19	CW-4-A18	S	S	S	S	S	S	S	S	nil	B1
20	CW-33-A18	S	S	S	S	S	S	S	S	nil	B1
21	CW-50-A18	S	S	S	S	S	S	S	S	nil	B1
22	CW-40-A18	S	S	S	S	S	S	S	S	nil	B1
23	CW-13-A18	S	S	S	S	S	S	S	S	nil	B1
24	CW-32-A18	S	S	S	S	S	S	S	S	nil	B1
25	CW-37-A18	S	S	S	S	S	S	S	S	nil	E

26	CW-29-A18	S	S	S	S	S	S	S	S	nil	B1
27	CW-21-A18	S	S	S	S	S	S	S	S	nil	B1
28	CW-18-A18	S	S	S	S	S	S	S	S	nil	E
29	CW-26-A18	S	S	S	S	S	S	S	S	nil	C
30	CW-24-A18	S	S	S	S	S	S	S	S	nil	B1
31	CW-30-A18	S	S	S	S	S	S	S	S	nil	B1
32	CW-17-A18	S	S	S	S	S	S	S	S	nil	B1
33	CW-45-A18	S	S	S	S	S	S	S	S	nil	B1
34	CW-19-A18	S	S	S	S	S	S	S	S	nil	B1
35	CW-12-A18	R	S	S	S	S	S	S	S	nil	C
36	CW-47-A18	S	S	S	S	S	S	S	S	nil	B1
37	**CW-49-A18**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**E**
38	CW-41-A18	S	S	S	S	S	S	S	S	nil	C
39	CW-11-A18	S	S	S	S	S	S	S	S	nil	B1

40	CW-5-A18	S	S	S	S	S	S	S	S	nil	B1
41	CW-51-A18	S	S	S	S	S	S	S	S	nil	B1
42	CW-52-A18	S	S	S	S	S	S	S	S	nil	B1
43	CW-34-A18	S	S	S	R	S	S	S	S	nil	B1
44	CW-22-A18	S	S	S	S	S	S	S	S	nil	B1
45	CW-35-A18	S	S	S	S	S	S	S	S	nil	E
46	CW-42-A18	S	S	S	S	S	S	S	S	nil	C
47	**CW-28-A18**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**B1**
48	CW-38-A18	S	S	S	S	S	S	S	S	nil	B1
49	CW-7-A18	S	S	S	S	S	S	S	S	nil	E

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing. Clearwaters organic dairy farm. Winter 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	CW-34-W-18	S	S	S	S	S	S	S	S	nil	E
2	CW-35-W18	S	S	S	S	S	S	S	S	nil	E
3	CW-37-W18	S	S	S	S	S	S	S	S	nil	B1
4	CW-29-W18	S	S	S	S	S	S	S	S	nil	E
5	CW-25-W18	S	S	S	S	S	S	S	S	nil	B1
6	CW-19-W18	S	S	S	S	S	S	S	S	nil	A
7	CW-7-W18	S	S	S	S	S	S	S	S	nil	B1
8	CW-35-W18	S	S	S	S	S	S	S	S	nil	B1
9	CW-21-W18	S	S	S	S	S	S	S	S	nil	B1
10	CW-28-W18	S	S	S	S	S	S	S	S	nil	E
11	CW-23-W18	S	S	S	S	S	S	S	S	nil	C

12	CW-22-W18	S	S	S	S	S	S	S	S	nil	B1
13	CW-30-W18	S	S	S	S	S	S	S	S	nil	B1
14	CW-31-W18	S	S	S	S	S	S	S	S	nil	B1
15	CW-32-W18	S	S	S	S	S	S	S	S	nil	B1
16	CW-24-W18	S	S	S	S	S	S	S	S	nil	B1
17	CW-8-W18	S	S	S	S	S	S	S	S	nil	B1
18	CW-27-W18	S	S	S	S	S	S	S	S	nil	E
19	CW-5-W18	S	S	S	S	S	S	S	S	nil	B1
20	CW-4-W18	S	S	S	S	S	S	S	S	nil	B1
21	CW-3-W18	S	S	S	S	S	S	S	S	nil	B1
22	CW-2-W18	S	S	S	S	S	S	S	S	nil	A
23	CW-12-W18	S	S	S	S	S	S	S	S	nil	B1
24	CW-13-W18	S	S	S	S	S	S	S	S	nil	B1
25	CW-26-W18	S	S	S	S	S	S	S	S	nil	C

26	CW-17-W18	S	S	S	S	S	S	S	S	nil	B1
27	CW-18-W18	S	S	S	S	S	S	S	S	nil	B1
28	CW-47-W18	S	S	S	S	S	S	S	S	nil	B1
29	CW-39-W18	S	S	S	S	S	S	S	S	nil	E
30	CW-41-W18	S	S	S	S	S	S	S	S	nil	D
31	CW-16-W18	S	S	S	S	S	S	S	S	nil	B1
32	CW-36-W18	S	S	S	S	S	S	S	S	nil	E
33	CW-15-W18	S	S	S	S	S	S	S	S	nil	CladeIII+IV +V
34	CW-48-W18	S	S	S	S	S	S	S	S	nil	E
35	CW-43-W18	S	S	S	S	S	S	S	S	nil	E
36	CW-40-W18	S	S	S	S	S	S	S	S	nil	B1
37	CW-45-W18	S	S	S	S	S	S	S	S	nil	B1
38	CW-10-W18	S	S	S	S	S	S	S	S	nil	B1
39	CW-33-W18	S	S	S	S	S	S	S	S	nil	E

40	CW-19-W18	S	S	S	S	S	S	S	S	nil	A
41	CW-44-W18	S	S	S	S	R	S	S	S	nil	A
42	CW-1-W18	S	S	S	S	S	S	S	S	nil	B1
43	CW-46-W18	S	S	S	S	R	S	S	S	nil	B1
44	CW-51-W18	S	S	S	S	S	S	S	S	nil	B1
45	CW-50-W18	S	S	S	S	S	S	S	S	nil	E
46	CW-49-W18	S	S	I	S	S	S	R	S	nil	E
47	CW-38-W18	S	S	S	S	S	S	S	S	nil	B1

Disc diffusion antimicrobial susceptibility testing. Mill Road conventional dairy farm. Spring 2017

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	MRD-34-S17	S	S	S	S	S	S	S	S	nil	B1
2	MRD-37-S17	R	S	S	S	S	S	S	S	nil	B1
3	MRD-29-S17	S	S	S	S	S	S	S	S	nil	B1
4	MRD-38-S17	S	S	S	S	S	S	S	S	nil	B1
5	MRD-19-S17	S	S	S	S	R	S	S	S	nil	B1
6	MRD-30-S17	S	S	I	S	S	S	S	S	nil	B1
7	**MRD-24-S17**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**B1**
8	MRD-35-S17	S	R	S	S	S	S	S	S	nil	B1
9	MRD-18-S17	S	S	S	S	S	S	S	S	nil	B1
10	MRD-27-S17	S	S	S	S	S	S	S	S	nil	C
11	MRD-33-S17	S	S	S	S	S	S	S	S	nil	B1

12	MRD-46-S17	S	S	S	S	S	S	S	S	nil	B1
13	MRD-48-S17	S	R	S	S	S	S	S	S	nil	B1
14	MRD-50-S17	S	S	S	S	S	S	S	S	nil	B1
15	MRD-16-S17	S	S	S	S	S	S	S	S	nil	A
16	MRD-13-S17	S	S	S	S	S	S	S	S	nil	B1
17	MRD-11-S17	S	S	S	S	S	S	S	S	nil	B1
18	MRD-9-S17	S	S	S	S	S	S	R	S	nil	B1
19	MRD-1-S17	S	S	R	S	R	S	S	S	nil	E
20	MRD-17-S17	S	R	S	S	S	S	S	S	nil	B1
21	MRD-15-S17	S	R	S	S	S	S	I	S	nil	B1
22	MRD-49-S17	S	S	S	S	R	S	S	S	nil	B1
23	MRD-40-S17	S	S	S	S	S	S	S	S	nil	B2
24	MRD-44-S17	S	S	S	S	S	S	S	S	nil	B1
25	MRD-2-S17	S	S	S	S	R	S	S	S	nil	B1

26	MRD-31-S17	S	S	S	S	S	S	S	S	nil	B1
27	MRD-21-S17	S	S	S	S	S	S	S	S	nil	B1
28	MRD-22-S17	S	S	S	S	S	S	S	S	nil	B1
29	MRD-12-S17	S	S	S	S	R	S	S	S	nil	B1
30	MRD-7-S17	S	S	S	S	S	S	S	S	nil	B1
31	MRD-5-S17	S	S	S	S	S	S	R	S	nil	B1
32	MRD-62-S17	S	S	S	S	S	S	S	S	nil	B1
33	MRD-59-S17	S	S	S	S	S	S	S	S	nil	B1
34	MRD-47-S17	S	S	S	S	S	S	S	S	nil	B1
35	MRD-61-S17	S	S	S	S	S	S	S	S	nil	B1
36	MRD-51-S17	S	S	I	S	S	S	S	S	nil	B1
37	MRD-60-S17	S	S	S	S	S	S	S	S	nil	B1
38	MRD-45-S17	S	S	S	S	S	S	S	S	nil	C
39	MRD-23-S17	S	S	S	S	S	S	S	S	nil	B2

40	MRD-58-S17	S	S	S	S	S	S	S	S	nil	B1
41	MRD-25-S17	S	S	S	S	S	S	S	S	nil	B1
42	MRD-10-S17	S	S	S	S	S	S	S	S	nil	B1
43	MRD-42-S17	S	I	S	S	R	S	S	S	nil	B1
44	MRD-37-S17	S	S	S	S	S	S	S	S	nil	B1
45	MRD-36-S17	S	S	S	S	S	S	S	S	nil	B1
46	MRD-57-S17	S	S	S	S	S	S	S	S	nil	B1
47	MRD-65-S17	S	S	S	S	S	S	S	S	nil	B1
48	MRD-13-S17	S	S	S	S	S	S	S	S	nil	A
49	MRD-41-S17	S	S	S	S	S	S	S	S	nil	B1
50	MRD-43-S17	S	I	S	S	R	S	S	S	nil	B1
51	MRD-32-S17	S	S	S	S	S	S	S	S	nil	B1
52	MRD-26-S17	S	S	S	S	S	S	S	S	nil	B1
53	MRD-35-S17	S	S	R	S	S	S	S	S	nil	B1

54	MRD-50-S17	S	S	S	S	S	S	S	S	nil	B1
55	MRD-54-S17	S	S	S	S	S	S	S	S	nil	B1
56	MRD-11-S17	S	S	S	S	S	S	S	S	nil	B1
57	MRD-28-S17	S	S	S	S	S	S	S	S	nil	A
58	MRD-20-S17	S	S	S	S	S	S	S	S	nil	B1
59	MRD-56-S17	S	S	S	S	S	S	S	S	nil	B1
60	MRD-53-S17	S	S	S	S	S	S	S	S	nil	E
61	MRD-8-S17	S	S	S	S	S	S	S	S	nil	E
62	MRD-40-S17	S	S	S	S	S	S	S	S	nil	E
63	MRD-44-S17	S	S	S	S	S	S	S	S	nil	C
64	MRD-31-S17	S	S	S	S	S	S	S	S	nil	A
65	MRD-24-S17	S	S	S	S	S	S	R	S	nil	B1
66	MRD-4-S17	S	I	S	S	R	S	S	S	nil	B1

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing. Mill Road conventional dairy farm. Spring 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	MRD-14-S18	S	S	S	S	S	S	S	S	nil	B1
2	MRD-15-S18	S	S	S	S	S	S	S	S	nil	B1
3	MRD-25-S18	S	S	S	S	S	S	S	S	nil	C
4	MRD-35-S18	S	S	S	S	S	S	S	S	nil	B1
5	MRD-4-S18	S	S	S	S	S	S	S	S	nil	B1
6	MRD-16-S18	S	S	S	S	S	S	S	S	nil	B1
7	MRD-18-S18	S	S	S	S	S	S	S	S	nil	B1
8	MRD-13-S18	S	S	S	S	S	S	S	S	nil	B1
9	MRD-21-S18	S	S	S	S	S	S	S	S	nil	B1
10	MRD-22-S18	S	S	S	S	S	S	S	S	nil	B1
11	MRD-23-S18	S	S	S	S	S	S	S	S	nil	B1

12	MRD-24-S18	S	S	S	S	S	S	S	S	nil	B1
13	MRD-12-S18	S	S	S	S	S	S	S	S	nil	B1
14	MRD-11-S18	S	S	S	S	S	S	S	S	nil	B1
15	MRD-19-S18	S	S	S	S	S	S	S	S	nil	B1
16	MRD-5-S18	S	S	S	S	S	S	S	S	nil	B1
17	MRD-1-S18	S	S	S	S	S	S	S	S	nil	B1
18	MRD-8-S18	S	S	S	S	S	S	S	S	nil	unknown
19	MRD-9-S18	S	S	S	S	S	S	S	S	nil	B1
20	MRD-10-S18	S	S	S	S	S	S	S	S	nil	B1
21	MRD-30-S18	S	S	S	S	S	S	S	S	nil	E
22	MRD-52-S18	S	S	S	S	S	S	S	R	nil	B1
23	MRD-34-S18	S	S	S	S	S	S	S	S	nil	B1
24	MRD-36-S18	R	S	S	S	S	S	S	S	nil	B1
25	MRD-38-S18	S	S	S	S	S	S	S	S	nil	E

26	MRD-49-S18	S	S	S	S	S	S	I	S	nil	B1
27	MRD-53-S18	S	S	S	S	S	S	S	R	nil	A
28	MRD-54-S18	S	I	I	S	S	S	S	I	nil	B1
29	MRD-55-S18	S	S	S	S	S	S	S	R	nil	B1
30	MRD-44-S18	S	S	S	S	S	S	S	S	nil	B1
31	MRD-47-S18	S	S	S	S	S	S	S	S	nil	C
32	MRD-45-S18	S	S	S	S	S	S	S	S	nil	B1
33	MRD-43-S18	S	S	R	S	S	S	S	S	nil	B1
34	MRD-39-S18	S	S	S	S	S	S	S	S	nil	B1
35	MRD-40-S18	S	S	S	S	S	S	S	S	nil	A
36	MRD-32-S18	S	S	S	S	S	S	S	S	nil	B1
37	MRD-46-S18	S	S	I	S	S	S	R	S	nil	unknown
38	MRD-29-S18	S	S	S	S	S	S	S	S	nil	B1
39	MRD-31-S18	S	S	S	S	S	S	S	S	nil	E

40	MRD-41-S18	S	S	S	S	S	S	S	S	nil	E
41	MRD-33-S18	S	S	S	S	S	S	S	S	nil	B1
42	MRD-42-S18	S	S	S	S	R	S	S	S	nil	B1
43	MRD-3-S18	S	S	S	S	S	S	S	S	nil	B1
44	MRD-2-S18	S	S	S	S	S	S	S	S	nil	C
45	MRD-17-S18	S	S	S	S	S	S	S	S	nil	B1
46	MRD-48-S18	S	S	S	S	S	S	S	S	nil	B2
47	MRD-6-S18	S	S	S	S	S	S	S	S	nil	B1
48	MRD-20-S18	S	S	S	S	S	S	S	S	nil	B1
49	MRD-27-S18	S	S	S	S	S	S	S	S	nil	B1
50	MRD-51-S18	S	S	S	S	S	S	S	S	nil	B1

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing. Mill Road conventional dairy farm. Autumn 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	MRD-61-A18	S	S	S	S	S	S	S	S	nil	A
2	MRD-43-A18	S	S	S	S	S	S	S	S	nil	B1
3	MRD-56-A18	S	S	S	S	S	S	S	S	nil	B1
4	MRD-62-A18	S	S	S	S	S	S	S	S	nil	B1
5	MRD-63-A18	S	S	S	S	S	S	S	S	nil	B1
6	MRD-64-A18	S	S	S	S	S	S	S	S	nil	B1
7	MRD-55-A18	S	S	S	S	S	S	S	S	nil	E
8	MRD-50-A18	S	S	S	S	S	S	S	S	nil	B1
9	MRD-44-A18	S	S	S	S	S	S	S	S	nil	B1
10	MRD-41-A18	S	S	S	S	S	S	S	S	nil	B1
11	MRD-52-A18	S	S	S	S	S	S	S	S	nil	A

12	MRD-59-A18	S	S	S	S	S	S	S	S	nil	B1
13	MRD-51-A18	S	S	S	S	S	S	S	S	nil	B1
14	MRD-53-A18	S	S	S	S	S	S	S	S	nil	B1
15	MRD-33-A18	S	S	S	S	S	S	S	S	nil	B1
16	MRD-38-A18	S	S	S	S	S	S	S	S	nil	B1
17	MRD-40-A18	S	S	R	S	S	S	S	S	nil	B1
18	MRD-37-A18	S	S	S	S	S	S	S	S	nil	E
19	MRD-47-A18	S	S	S	S	S	S	S	S	nil	B1
20	MRD-49-A18	S	S	S	S	S	S	S	S	nil	cladeI
21	MRD-46-A18	S	S	S	S	S	S	S	S	nil	B1
22	MRD-48-A18	S	S	S	S	S	S	S	S	nil	B1
23	MRD-45-A18	S	S	S	S	S	S	S	S	nil	B1
24	MRD-1-A18	S	S	S	S	S	S	S	S	nil	B1
25	MRD-10-A18	S	S	S	S	S	S	S	S	nil	B1

26	MRD-35-A18	S	S	S	S	S	S	S	S	nil	cladeIII+IV+V
27	MRD-2-A18	S	S	S	S	S	S	S	S	nil	A
28	MRD-7-A18	S	S	S	S	S	S	S	S	nil	B1
29	MRD-30-A18	S	S	S	S	S	S	S	S	nil	B1
30	MRD-31-A18	S	S	S	S	S	S	S	S	nil	B1
31	MRD-6-A18	S	S	S	S	S	S	S	S	nil	B1
32	MRD-8-A18	S	S	S	S	S	S	S	S	nil	B1
33	MRD-3-A18	S	S	S	S	S	S	S	S	nil	A
34	MRD-18-A18	S	S	S	S	S	S	S	S	nil	B1
35	MRD-16-A18	S	S	S	S	S	S	S	S	nil	B1
36	MRD-17-A18	S	S	S	S	S	S	S	S	nil	B1
37	MRD-19-A18	S	S	S	S	S	S	S	S	nil	B1
38	MRD-34-A18	S	S	S	S	S	S	S	S	nil	B1
39	MRD-32-A18	S	S	S	S	S	S	S	S	nil	B1

40	MRD-25-A18	S	S	S	S	S	S	S	S	nil	B1
41	MRD-15-A18	S	S	S	S	S	S	R	S	nil	E
42	MRD-29-A18	S	S	S	S	S	S	S	S	nil	B1
43	MRD-26-A18	S	S	S	S	S	S	S	S	nil	E
44	MRD-27-A18	S	S	S	S	S	S	S	S	nil	D
45	MRD-22-A18	S	S	S	S	S	S	S	S	nil	B1
46	MRD-58-A18	S	S	S	S	S	S	S	S	nil	B1
47	MRD-28-A18	S	S	S	S	S	S	S	S	nil	B1
48	**MRD-21-A18**	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**AmpC+**	**B1**
49	MRD-54-A18	S	S	S	S	S	S	S	S	nil	B2
50	MRD-39-A18	S	S	R	S	S	S	R	S	nil	B1
51	MRD-42-A18	S	S	S	S	S	S	R	S	nil	B1
52	MRD-57-A18	S	S	S	S	S	S	R	S	nil	B1
53	MRD-5-A18	S	S	S	S	S	S	R	S	nil	E

54	MRD-13-A18	S	S	S	S	S	S	R	S	nil	B1
55	MRD-14-A18	S	S	S	S	S	S	R	S	nil	B1

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing. Mill Road conventional dairy farm. Autumn 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	MRD-16-W18	S	S	S	S	S	S	S	S	nil	B1
2	MRD-19-W18	S	S	S	S	S	S	S	S	nil	A
3	MRD-6-W18	S	S	S	S	S	S	S	S	nil	unknown
4	MRD-18-W18	S	S	S	S	S	S	S	S	nil	A
5	MRD-15-W18	S	S	S	S	S	S	S	S	nil	B1
6	MRD-36-W18	S	S	S	S	S	S	S	S	nil	B1
7	MRD-1-W18	S	S	S	S	S	S	S	S	nil	B1
8	MRD-39-W18	S	S	S	S	S	S	S	S	nil	B1
9	MRD-50-W18	S	S	S	S	S	S	S	S	nil	B1
10	MRD-41-W18	S	S	S	S	S	S	S	S	nil	unknown
11	MRD-55-W18	S	S	S	S	S	S	S	S	nil	B1

12	MRD-25-W18	S	S	S	S	S	S	S	S	nil	E
13	MRD-38-W18	S	S	S	S	S	S	S	S	nil	B1
14	MRD-44-W18	S	S	S	S	S	S	S	S	nil	B1
15	MRD-43-W18	S	S	S	S	S	S	S	S	nil	B1
16	MRD-47-W18	S	S	S	S	S	S	S	S	nil	E
17	MRD-42-W18	S	S	S	S	S	S	S	S	nil	B1
18	MRD-46-W18	S	S	S	S	S	S	S	S	nil	E
19	MRD-30-W18	S	S	S	S	S	S	S	S	nil	B1
20	MRD-31-W18	S	S	S	S	S	S	S	S	nil	E
21	MRD-27-W18	S	S	S	S	S	S	S	S	nil	unknown
22	MRD-48-W18	S	S	S	S	S	S	S	S	nil	B1
23	MRD-49-W18	S	S	S	S	S	S	S	S	nil	B1
24	MRD-26-W18	S	S	S	S	S	S	S	S	nil	E
25	MRD-20-W18	S	S	S	S	S	S	S	S	nil	A

26	MRD-29-W18	S	S	S	S	S	S	S	S	nil	B1
27	MRD-37-W18	S	S	S	S	S	S	S	S	nil	B1
28	MRD-35-W18	S	S	S	S	S	S	S	S	nil	B1
29	MRD-13-W18	S	S	S	S	S	S	S	S	nil	A
30	MRD-9-W18	S	S	S	S	S	S	S	S	nil	B1
31	MRD-5-W18	S	S	S	S	S	S	S	S	nil	A
32	MRD-7-W18	S	S	S	S	S	S	S	S	nil	A
33	MRD-14-W18	S	S	S	S	S	S	S	S	nil	A
34	MRD-10-W18	S	S	S	S	S	S	S	S	nil	A
35	MRD-8-W18	S	S	S	S	S	S	S	S	nil	A
36	MRD-12-W18	S	S	S	S	S	S	R	S	nil	B1
37	MRD-17-W18	S	S	S	S	S	S	S	S	nil	B1
38	MRD-11-W18	S	S	S	S	S	S	S	S	nil	B1
39	MRD-40-W18	S	S	I	S	S	S	I	R	nil	E

40	MRD-24-W18	S	S	I	I	S	S	I	I	nil	B1
41	**MRD-22-W18**	**R**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**B1**
42	MRD-45-W18	S	S	S	S	S	S	S	S	nil	B1
43	MRD-3-W18	S	S	S	S	S	S	R	S	nil	B1
44	MRD-34-W18	S	S	I	S	S	S	I	I	nil	B1
45	MRD-4-W18	S	S	S	S	S	S	S	S	nil	B1
46	MRD-23-W18	S	S	S	S	S	S	R	S	nil	B1
47	MRD-2-W18	S	S	S	S	S	S	R	S	nil	B1
48	MRD-32-W18	S	S	I	S	S	S	I	S	nil	B1
49	MRD-21-W18	S	S	S	S	S	S	R	S	nil	B1
50	MRD-33-W18	S	S	I	S	S	S	I	I	nil	B1

Isolate in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing. Peel Forest conventional dairy farm. Spring 2017

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	PF-6-S17	S	S	S	S	S	S	S	S	nil	C
2	PF-9-S17	R	S	S	S	S	S	S	R	nil	C
3	PF-8-S17	S	S	S	S	S	S	S	S	nil	C
4	PF-28-S17	S	S	S	S	S	S	S	S	nil	B1
5	PF-30-S17	S	S	S	R	S	S	S	S	nil	C
6	PF-31-S17	S	S	S	R	S	S	S	S	nil	B1
7	PF-29-S17	S	S	S	S	S	S	S	S	nil	C
8	PF-35-S17	S	R	S	S	S	S	S	S	nil	B1
9	PF-1-S17	R	S	S	S	S	S	S	S	nil	B1
10	PF-2-S17	R	S	S	S	S	S	S	S	nil	B1
11	PF-27-S17	S	S	S	R	S	S	S	S	nil	C

12	PF-43-S17	S	S	S	S	S	S	S	S	nil	B1
13	PF-46-S17	S	S	S	R	S	S	S	S	nil	B1
14	PF-39-S17	S	S	S	S	S	S	S	S	nil	E
15	PF-45-S17	S	S	R	S	S	S	S	S	nil	CladeI+II
16	PF-49-S17	S	S	S	S	S	S	S	S	nil	B1
17	PF-47-S17	S	S	S	R	S	S	S	S	nil	B1
18	PF-48-S17	S	S	S	R	S	S	S	S	nil	C
19	PF-36-S17	S	S	R	S	S	S	S	S	nil	B1
20	PF-37-S17	S	S	S	S	S	S	S	R	nil	B1
21	PF-38-S17	S	S	S	S	S	S	S	S	nil	C
22	PF-25-S17	S	S	S	S	S	S	S	S	nil	B1
23	PF-44-S17	S	S	S	S	S	S	S	S	nil	B1
24	PF-22-S17	S	R	S	S	S	S	S	S	nil	B1
25	PF-19-S17	R	S	S	R	S	S	S	S	nil	C

26	PF-18-S17	S	R	S	S	S	S	S	S	nil	A
27	PF-16-S17	R	S	S	R	S	S	S	S	nil	B1
28	PF-33-S17	S	S	S	S	S	S	S	S	nil	C
29	PF-26-S17	S	S	S	S	S	S	S	S	nil	C
30	PF-24-S17	R	S	S	S	S	S	S	S	nil	C
31	PF-23-S17	S	S	S	S	S	S	S	S	nil	B1
32	PF-15-S17	S	S	S	S	S	S	S	S	nil	B1
33	PF-13-S17	S	S	S	S	S	S	S	S	nil	B1
34	PF-5-S17	S	S	R	S	S	S	S	S	nil	C
35	PF-20-S17	S	S	S	S	S	S	R	S	nil	E
36	PF-41-S17	S	S	S	R	S	S	S	S	nil	E
37	PF-4-S17	S	S	S	S	S	S	S	S	nil	E
38	PF-40-S17	R	S	I	I	I	S	S	S	nil	B1
39	PF-21-S17	R	S	S	S	S	S	S	S	nil	B1

40	PF-42-S17	S	S	S	S	R	S	S	S	nil	E
41	PF-50-S17	S	S	S	S	R	S	S	S	nil	B1
42	PF-34-S17	S	S	S	S	S	S	S	S	nil	C
43	PF-17-S17	S	S	S	S	S	S	R	S	nil	C
44	PF-7-S17	S	S	S	S	S	S	S	S	nil	B1
45	PF-10-S17	S	S	R	S	S	S	S	S	nil	B1
46	PF-32-S17	R	S	S	S	S	S	S	S	nil	E
47	PF-14-S17	S	R	S	S	S	S	S	S	nil	B1
48	PF-11-S17	S	S	S	S	S	S	S	S	nil	B1
49	PF-12-S17	S	S	S	S	S	S	S	S	nil	B1
50	PF-51-S17	S	S	S	S	S	S	S	S	nil	B1

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing. Peel Forest conventional dairy farm. Spring 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	PF-49-S18	R	S	S	S	S	S	S	S	nil	B1
2	PF-48-S18	S	S	S	S	S	S	S	S	nil	E
3	PF-51-S18	R	S	S	S	S	S	S	S	nil	B1
4	PF-52-S18	R	S	I	S	S	S	S	S	nil	B1
5	PF-55-S18	S	S	S	S	S	S	S	S	nil	A
6	PF-40-S18	S	S	S	S	S	S	S	S	nil	B1
7	PF-46-S18	R	S	S	S	S	S	S	S	nil	B1
8	PF-42-S18	R	S	S	S	S	S	S	S	nil	B1
9	**PF-25-S18**	**R**	**S**	**I**	**S**	**S**	**S**	**R**	**S**	**AmpC**	**B1**
10	PF-36-S18	S	S	S	S	S	S	S	S	nil	B1
11	PF-56-S18	S	S	S	S	S	S	S	S	nil	C

12	PF-1-S18	S	S	S	S	S	S	S	S	nil	B1
13	PF-4-S18	S	S	S	S	S	S	S	S	nil	B1
14	PF-15-S18	S	S	S	S	S	S	S	S	nil	B1
15	PF-2-S18	S	S	S	S	S	S	S	S	nil	B1
16	PF-3-S18	S	S	S	S	S	S	S	S	nil	B1
17	PF-7-S18	S	S	S	S	S	S	S	S	nil	B1
18	PF-10-S18	S	S	S	S	S	S	S	S	nil	B1
19	PF-14-S18	S	S	S	S	S	S	S	S	nil	B1
20	PF-13-S18	S	S	S	S	S	S	S	S	nil	B1
21	PF-16-S18	S	S	S	S	S	S	S	S	nil	B1
22	PF-11-S18	S	S	S	S	S	S	S	S	nil	B1
23	PF-12-S18	S	S	S	S	S	S	S	S	nil	B1
24	PF-6-S18	S	S	S	S	S	S	S	S	nil	B1
25	PF-5-S18	S	S	S	S	S	S	S	S	nil	A

26	PF-8-S18	S	S	S	S	S	S	S	S	nil	B1
27	PF-17-S18	S	S	S	S	S	S	S	S	nil	B1
28	PF-24-S18	S	S	S	S	S	S	S	S	nil	B1
29	PF-23-S18	S	S	S	S	S	S	S	S	nil	B1
30	PF-22-S18	S	S	S	S	S	S	S	S	nil	B1
31	PF-21-S18	S	S	S	S	S	S	S	S	nil	B1
32	PF-18-S18	S	S	S	S	S	S	S	S	nil	B1
33	PF-19-S18	S	S	S	S	S	S	S	S	nil	E
34	PF-33-S18	S	S	S	S	S	S	S	S	nil	B1
35	PF-38-S18	S	S	S	S	S	S	S	S	nil	B1
36	PF-50-S18	S	S	S	S	S	S	S	S	nil	B1
37	PF-41-S18	S	S	S	S	S	S	S	S	nil	E
38	PF-29-S18	S	S	S	S	S	S	S	S	nil	E
39	PF-27-S18	S	S	S	S	S	S	S	S	nil	A

40	PF-45-S18	S	S	S	S	S	S	S	S	nil	B1
41	PF-37-S18	S	S	S	S	S	S	S	S	nil	E
42	PF-31-S18	S	S	S	S	S	S	R	S	nil	B1
43	PF-30-S18	S	S	S	S	S	S	S	S	nil	B1
44	PF-43-S18	S	S	S	S	S	S	S	S	nil	B1
45	PF-47-S18	R	S	S	S	S	S	S	S	nil	B1
46	PF-28-S18	S	S	S	S	S	S	S	S	nil	B1
47	PF-53-S18	S	S	S	S	S	S	S	S	nil	B1
48	PF-20-S18	S	S	S	S	S	S	S	S	nil	cladeIII+IV+V
49	PF-26-S18	S	S	S	S	S	S	S	S	nil	E

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing. Peel Forest conventional dairy farm. Autumn 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
2	PF-29-A18	S	S	S	S	S	S	S	S	nil	B1
3	PF-34-A18	S	S	S	S	S	S	S	S	nil	B1
4	PF-1-A18	S	S	S	S	S	S	S	S	nil	B1
5	PF-3-A18	S	S	S	S	S	S	S	S	nil	B1
6	PF-28-A18	S	S	S	S	S	S	S	S	nil	B1
7	PF-27-A18	S	S	S	S	S	S	S	S	nil	B1
8	PF-8-A18	S	S	S	S	S	S	S	S	nil	B1
9	PF-54-A18	S	S	S	S	S	S	S	S	nil	C
10	PF-43-A18	S	S	S	S	S	S	S	S	nil	D
11	PF-57-A18	S	S	S	S	S	S	S	S	nil	E
12	PF-55-A18	S	S	S	S	S	S	S	S	nil	E

13	PF-38-A18	S	S	S	S	S	S	S	S	nil	B1
14	PF-47-A18	S	S	S	S	S	S	S	S	nil	B1
15	PF-52-A18	S	S	S	S	S	S	S	S	nil	B1
16	PF-49-A18	S	S	S	S	S	S	S	S	nil	B1
17	PF-51-A18	S	S	S	S	S	S	S	S	nil	C
18	PF-13-A18	S	S	S	S	S	S	S	S	nil	B1
19	PF-2-A18	S	S	S	S	S	S	S	S	nil	B1
20	PF-20-A18	S	S	S	S	S	S	S	S	nil	B1
21	**PF-32-A18**	**R**	**S**	**I**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**B1**
22	PF-6-A18	S	S	S	S	S	S	S	S	nil	B1
23	PF-33-A18	S	S	S	S	S	S	S	S	nil	B1
24	PF-42-A18	S	S	S	S	S	S	S	S	nil	B1
25	PF-26-A18	S	S	S	S	S	S	S	S	nil	B1
26	PF-12-A18	S	S	S	S	S	S	S	S	nil	E

27	PF-37-A18	S	S	S	S	S	S	S	S	nil	B1
28	PF-40-A18	S	S	S	S	S	S	S	S	nil	E
29	PF-7-A18	S	S	S	S	S	S	S	S	nil	B1
30	PF-56-A18	R	S	S	S	S	S	S	S	nil	B1
31	PF-5-A18	S	S	S	S	S	S	S	S	nil	B1
32	PF-11-A18	S	S	S	S	S	S	S	S	nil	E
33	PF-4-A18	S	S	S	S	S	S	S	S	nil	B1
34	PF-16-A18	S	S	S	S	S	S	S	S	nil	B1
35	**PF-30-A18**	**S**	**S**	**I**	**S**	**S**	**S**	**R**	**S**	**AmpC+**	**B1**
36	PF-60-A18	S	S	S	S	S	S	S	S	nil	B1
37	PF-10-A18	S	S	S	S	S	S	S	S	nil	cladeIII +IV+V
38	PF-63-A18	S	S	S	S	S	S	S	S	nil	B1
39	PF-31-A18	S	S	S	S	S	S	S	S	nil	B1
40	PF-61-A18	S	S	S	S	S	S	S	S	nil	B1

41	PF-62-A18	S	S	S	S	S	S	S	S	nil	B1
42	**PF-15-A18**	**R**	**S**	**S**	**R**	**S**	**S**	**R**	**S**	**ESBL**	**B1**
43	PF-23-A18	S	S	S	S	S	S	S	S	nil	cladeIII +IV+V
44	PF-9-A18	S	S	S	S	S	S	R	S	nil	C
45	PF-58-A18	S	S	R	S	S	S	R	S	nil	B1
46	PF-24-A18	S	S	S	S	S	S	R	S	nil	B1
47	PF-19-A18	S	S	S	S	S	S	S	S	nil	E
48	**PF-14-A18**	**R**	**S**	**S**	**R**	**S**	**S**	**R**	**S**	**AmpC+**	**B1**
49	PF-22-A18	S	S	S	S	S	S	R	S	nil	E
50	PF-41-A18	S	S	S	S	S	S	R	S	nil	B1
51	PF-21-A18	S	S	S	S	S	S	S	S	nil	B1
52	PF-44-A18	S	S	S	S	S	S	R	S	nil	B1
53	PF-35-A18	R	S	I	S	S	S	S	S	nil	B1

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing. Peel Forest conventional dairy farm. Winter 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
2	PF-15-W18	S	S	S	S	S	S	S	S	nil	B1
3	PF-14-W18	S	S	S	S	S	S	S	S	nil	B1
4	PF-4-W18	S	S	S	S	S	S	R	S	nil	B1
5	PF-1-W18	S	S	S	S	S	S	S	S	nil	B1
6	PF-5-W18	S	S	S	S	S	S	S	S	nil	B1
7	PF-8-W18	S	S	S	S	S	S	S	S	nil	B1
8	PF-16-W18	S	S	S	S	S	S	S	S	nil	B1
9	PF-11-W18	S	S	S	S	S	S	S	S	nil	B1
10	PF-13-W18	S	S	S	S	S	S	S	S	nil	C
11	PF-10-W18	S	S	S	S	S	S	S	S	nil	B1

12	PF-9-W18	S	S	S	S	S	S	R	S	nil	B1
13	PF-3-W18	S	S	S	S	S	S	S	S	nil	B1
14	PF-2-W18	S	S	S	S	S	S	S	S	nil	B1
15	PF-50-W18	S	S	S	S	S	S	S	S	nil	B1
16	PF-51-W18	S	S	S	S	S	S	S	S	nil	B1
17	PF-43-W18	S	S	S	S	S	S	S	S	nil	B1
18	PF-34-W18	S	S	S	S	S	S	S	S	nil	B1
19	PF-53-W18	S	S	S	S	S	S	S	S	nil	A
20	PF-56-W18	S	S	S	S	S	S	S	S	nil	A
21	PF-49-W18	S	S	S	S	S	S	S	S	nil	B1
22	PF-48-W18	R	S	S	S	S	S	S	S	nil	B1
23	PF-35-W18	S	S	S	S	S	S	S	S	nil	B1
24	PF-46-W18	S	S	S	S	S	S	S	S	nil	B1
25	PF-38-W18	S	S	S	S	S	S	S	S	nil	A

26	PF-36-W18	S	S	S	S	S	S	S	S	nil	B1
27	PF-33-W18	S	S	S	S	S	S	S	S	nil	B1
28	PF-21-W18	S	S	S	S	S	S	S	R	nil	B1
29	PF-20-W18	S	S	S	S	S	S	S	S	nil	B1
30	PF-19-W18	S	S	S	S	S	S	S	S	nil	B1
31	PF-22-W18	S	S	S	S	S	S	S	S	nil	A
32	PF-18-W18	S	S	S	S	S	S	S	S	nil	E
33	PF-25-W18	S	S	S	S	S	S	S	S	nil	B1
34	PF-29-W18	S	S	S	S	S	S	S	S	nil	A
35	PF-28-W18	S	S	S	S	S	S	S	S	nil	B1
36	PF-30-W18	S	S	S	S	S	S	S	S	nil	B1
37	PF-26-W18	S	S	S	S	S	S	S	S	nil	B1
38	PF-32-W18	S	S	S	S	S	S	S	S	nil	B1
39	PF-31-W18	S	S	S	S	S	S	S	S	nil	B1

40	PF-63-W18	S	S	S	S	S	S	S	S	nil	B1
41	PF-27-W18	S	S	S	S	S	S	S	R	nil	B1
42	**PF-45-W18**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**AmpC**	**E**
43	PF-37-W18	S	S	S	S	S	S	R	S	nil	B1
44	PF-12-W18	S	S	I	S	S	S	I	S	nil	B1
45	**PF-52-W18**	**S**	**S**	**R**	**S**	**S**	**S**	**R**	**S**	**AmpC**	**B1**
46	PF-42-W18	S	S	S	S	S	S	R	S	nil	B1
47	PF-39-W18	S	S	S	S	S	S	R	S	nil	B1
48	**PF-55-W18**	**R**	**S**	**I**	**R**	**S**	**S**	**R**	**S**	**ESBL**	**A**

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing Totara Valley organic dairy farm. Spring 2017

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
2	TL-9-S17	S	S	S	S	S	S	S	S	nil	B1
3	TL-3-S17	S	S	S	S	S	S	S	S	nil	B1
4	TL-27-S17	S	S	S	S	S	S	S	S	nil	Unknown
5	TL-29-S17	S	S	R	S	S	S	R	S	nil	B1
6	TL-31-S17	S	S	R	S	S	S	R	S	nil	B1
7	TL-46-S17	S	S	S	S	S	S	R	S	nil	B1
8	TL-49-S17	S	S	S	S	S	S	S	S	nil	B1
9	TL-35-S17	S	S	R	S	S	S	R	S	nil	B1
10	TL-34-S17	S	S	R	S	S	S	S	S	nil	E
11	TL-24-S17	S	S	S	S	S	S	S	S	nil	B1

12	TL-12-S17	S	S	S	S	S	S	S	S	nil	B1
13	TL-21-S17	S	S	S	S	S	S	S	S	nil	B1
14	TL-17-S17	S	S	S	S	S	S	R	S	nil	B1
15	TL-4-S17	S	S	S	S	S	S	S	S	nil	B1
16	TL-7-S17	S	S	S	S	S	S	S	S	nil	B1
17	TL-32-S17	S	S	S	S	S	S	S	S	nil	E
18	TL-19-S17	S	S	R	S	S	S	R	S	nil	Unknown
19	TL-26-S17	S	S	S	S	S	S	S	S	nil	Unknown
20	TL-22-S17	S	S	R	S	S	S	R	S	nil	Unknown
21	TL-18-S17	S	S	S	S	S	S	S	S	nil	B1
22	TL-10-S17	S	S	S	S	S	S	R	S	nil	B1
23	TL-36-S17	S	S	S	S	S	S	S	S	nil	B1
24	TL-1-S17	S	S	R	S	S	S	R	S	nil	B1
25	TL-16-S17	S	S	S	S	S	S	S	S	nil	B1

26	TL-44-S17	S	S	R	S	S	S	R	S	nil	Unknown
27	TL-6-S17	S	S	S	S	S	S	S	S	nil	B1
28	TL-28-S17	S	S	S	S	S	S	S	S	nil	B1
29	TL-5-S17	S	S	S	S	S	S	S	S	nil	C
30	TL-40-S17	S	S	S	S	S	S	S	S	nil	E
31	TL-45-S17	S	S	S	S	S	S	S	S	nil	B1
32	TL-23-S17	S	S	S	S	S	S	S	S	nil	B1
33	TL-14-S17	S	S	S	S	S	S	S	S	nil	C
34	TL-38-S17	S	S	S	S	S	S	S	S	nil	B1
35	TL-20-S17	S	S	S	S	S	S	R	S	nil	B1
36	TL-50-S17	S	S	S	S	S	S	R	S	nil	Unknown
37	TL-11-S17	S	S	S	S	S	S	S	S	nil	C
38	TL-41-S17	S	S	S	S	S	S	S	S	nil	B1
39	TL-49-S17	S	S	S	S	S	S	S	S	nil	B1

40	TL-43-S17	S	S	S	S	S	S	S	S	nil	B1
41	TL-47-S17	R	S	S	S	S	S	R	S	nil	B1
42	TL-37-S17	S	S	S	S	S	S	R	S	nil	Unknown
43	TL-42-S17	S	S	R	S	S	S	R	S	nil	E
44	TL-2-S17	S	S	R	S	S	S	R	S	nil	C
45	TL-51-S17	S	S	S	S	S	S	R	S	nil	B1
46	TL-21-S17	S	S	S	S	S	S	S	S	nil	B1
47	TL-39-S17	S	S	S	S	S	S	S	S	nil	C
48	TL-25-S17	S	S	S	S	S	S	S	S	nil	B1
49	TL-8-S17	S	S	S	S	S	S	R	S	nil	unknown
50	TL-57-S17	S	S	S	S	S	S	S	S	nil	B1

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing Totara Valley organic dairy farm. Spring 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	TL-30-S18	S	S	S	S	S	S	S	S	nil	B1
2	TL-34-S18	S	S	S	S	S	S	S	S	nil	B1
3	TL-2-S18	S	S	S	S	S	S	S	S	nil	B1
4	TL-27-S18	S	S	S	S	S	S	S	S	nil	B1
5	TL-17-S18	S	S	S	S	S	S	S	S	nil	B1
6	TL-16-S18	S	S	S	S	S	S	S	S	nil	B1
7	TL-23-S18	S	S	S	S	S	S	S	S	nil	B1
8	TL-24-S18	S	S	S	S	S	S	S	S	nil	B1
9	TL-12-S18	S	S	I	S	S	S	S	S	nil	B1
10	TL-28-S18	S	S	S	S	S	S	S	S	nil	A
11	TL-26-S18	S	S	S	S	S	S	S	S	nil	B1

12	TL-32-S18	S	S	R	S	S	S	S	S	nil	B1
13	TL-45-S18	S	S	S	S	S	S	S	S	nil	E
14	TL-46-S18	S	S	S	S	S	S	S	S	nil	B1
15	TL-47-S18	S	S	S	S	S	S	S	S	nil	B1
16	TL-31-S18	S	S	S	S	S	S	S	S	nil	CladeIII+IV + V
17	**TL-56-S18**	**S**	**S**	**I**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**B1**
18	TL-30-S18	S	S	S	S	S	S	S	S	nil	B1
19	TL-29-S18	S	S	S	S	S	S	S	S	nil	B1
20	TL-42-S18	S	S	S	S	S	S	S	S	nil	B1
21	TL-52-S18	S	S	S	S	S	S	S	S	nil	B1
22	TL-49-S18	S	S	S	S	S	S	S	S	nil	B1
23	TL-50-S18	S	S	S	S	S	S	S	S	nil	B1
24	TL-51-S18	S	S	S	S	S	S	S	S	nil	E
25	TL-41-S18	S	S	S	S	S	S	S	S	nil	B1

26	TL-44-S18	S	S	S	S	S	S	S	S	nil	B1
27	TL-42-S18	S	S	S	S	S	S	S	S	nil	B1
28	TL-37-S18	S	S	S	S	S	S	S	S	nil	B1
29	TL-36-S18	S	S	S	S	S	S	S	S	nil	B1
30	TL-43-S18	S	S	S	S	S	S	S	S	nil	B1
31	TL-8-S18	S	S	S	S	S	S	S	S	nil	B1
32	TL-9-S18	S	S	S	S	S	S	S	S	nil	B1
33	TL-6-S18	S	S	S	S	S	S	S	S	nil	B1
34	TL-7-S18	S	S	S	S	S	S	S	S	nil	B1
35	TL-18-S18	S	S	S	S	S	S	S	S	nil	B1
36	TL-12-S18	S	S	S	S	S	S	S	S	nil	B1
37	TL-11-S18	S	S	S	S	S	S	S	S	nil	B1
38	TL-10-S18	S	S	S	S	S	S	S	S	nil	B1
39	TL-20-S18	S	S	S	S	S	S	S	S	nil	B1

40	TL-4-S18	S	S	S	S	S	S	S	S	nil	B1
41	TL-5-S18	S	S	S	S	S	S	S	S	nil	B1
42	TL-3-S18	S	S	S	S	S	S	S	S	nil	B1
43	TL-21-S18	S	S	S	S	S	S	S	S	nil	B1
44	TL-14-S18	S	S	S	S	S	S	S	S	nil	B1
45	TL-48-S18	S	S	S	S	S	S	S	S	nil	B1
46	TL-53-S18	S	S	R	S	S	S	S	S	nil	B1
47	TL-19-S18	S	S	S	S	S	S	S	S	nil	B1
48	TL-38-S18	S	S	S	S	S	S	S	S	nil	B1
49	TL-55-S18	S	S	S	S	S	S	S	S	nil	B1
50	**TL-54-S18**	**S**	**S**	**I**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**B1**
51	**TL-1-S18**	**S**	**S**	**I**	**S**	**R**	**S**	**R**	**S**	**AmpC+**	**B1**

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing Totara Valley organic dairy farm. Autumn 2018

#	DfSEC	Te30	Na30	CPD10	CN10	MEM10	CIP30	FOX30	C30	Phenotype screening	Phylogenetic group
1	TL-31-A18	S	S	S	S	S	S	S	S	nil	B1
2	TL-29-A18	S	S	S	S	S	S	S	S	nil	B1
3	TL-22-A18	S	S	S	S	S	S	S	S	nil	unknown
4	TL-15-A18	S	S	S	S	S	S	S	S	nil	B1
5	TL-10-A18	S	S	S	S	S	S	S	S	nil	unknown
6	TL-47-A18	S	S	S	S	S	S	S	S	nil	B1
7	TL-39-A18	S	S	S	S	S	S	S	S	nil	B1
8	**TL-12-A18**	**S**	**S**	**S**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**B1**
9	TL-27-A18	S	S	I	S	S	S	S	S	nil	B1
10	TL-7-A18	S	S	S	S	S	S	S	S	nil	B1
11	TL-9-A18	S	S	S	S	S	S	S	S	nil	unknown
12	TL-36-A18	S	S	S	S	S	S	R	S	nil	unknown
13	TL-40-A18	S	S	S	S	S	S	S	S	nil	unknown
14	TL-18-A18	S	S	S	S	S	S	S	S	nil	A
15	TL-38-A18	S	S	S	S	S	S	S	S	nil	B1
16	TL-20-A18	S	S	S	S	S	S	S	S	nil	B1
17	TL-34-A18	S	S	S	S	S	S	R	S	nil	unknown
18	TL-42-A18	S	S	S	S	I	S	S	S	nil	B1
19	TL-32-A18	S	S	S	R	S	S	S	S	nil	B1
20	TL-26-A18	S	S	S	S	S	S	R	S	nil	unknown
21	TL-43-A18	S	S	S	S	S	S	S	S	nil	B1
22	TL-33-A18	S	S	S	S	S	S	R	S	nil	B1

23	TL-19-A18	S	S	S	S	S	S	S	S	nil	B1
24	TL-25-A18	S	S	S	S	S	S	S	S	nil	B1
25	TL-1-A18	S	S	S	S	S	S	S	S	nil	A
26	TL-11-A18	S	S	S	R	S	S	S	S	nil	B1
27	TL-24-A18	S	S	S	S	S	S	S	S	nil	A
28	TL-16-A18	S	S	S	S	S	S	S	S	nil	B1
29	TL-14-A18	S	S	S	R	S	S	S	S	nil	B1
30	TL-48-A18	S	S	S	S	S	S	S	S	nil	B1
31	TL-52-A18	S	S	S	S	S	S	S	S	nil	B1
32	TL-50-A18	S	S	S	S	S	S	S	S	nil	B1
33	TL-13-A18	S	S	S	S	S	S	S	S	nil	B1
34	TL-17-A18	S	S	S	S	S	S	R	S	nil	B1
35	TL-45-A18	S	S	S	S	S	S	S	S	nil	B1
36	TL-53-A18	S	S	S	S	S	S	S	S	nil	B1
37	TL-54-A18	S	S	S	S	S	S	S	S	nil	B1
38	TL-49-A18	S	S	I	S	S	S	R	S	nil	B1
39	TL-46-A18	S	S	S	S	S	S	S	S	nil	B1
40	TL-55-A18	S	S	S	S	S	S	S	S	nil	B1
41	TL-41-A18	S	S	S	S	S	S	S	S	nil	B1
42	TL-21-A18	S	S	S	S	S	S	S	S	nil	B1
43	TL-35-A18	S	S	S	S	S	S	S	S	nil	B1
44	**TL-87-A18**	**S**	**I**	**S**	**S**	**S**	**S**	**R**	**S**	**ESBL**	**B1**
45	TL-28-A18	S	S	S	S	S	S	S	S	nil	CladeI + II
46	TL-44-A18	S	S	S	S	S	S	R	S	nil	B1
47	**TL-2-A18**	**S**	**S**	**S**	**I**	**S**	**S**	**R**	**S**	**ESBL**	B1
48	TL-56-A18	S	S	R	S	S	S	R	S	nil	CladeI + II

Isolates in bold showed ≤10 mm inhibition zone to FOX30

Disc diffusion antimicrobial susceptibility testing Totara Valley organic dairy farm. Winter 2018											
#	**DfSEC**	**Te30**	**Na30**	**CPD10**	**CN10**	**MEM10**	**CIP30**	**FOX30**	**C30**	**Phenotype screening**	**Phylogenetic group**
2	TL-10-W18	S	S	S	S	S	S	S	S	nil	B1
3	TL-12-W18	S	S	S	S	S	S	S	S	nil	B1
4	TL-23-W18	S	S	S	S	S	S	S	S	nil	A
5	TL-3-W18	S	S	S	S	S	S	S	S	nil	A
6	TL-8-W18	S	S	S	S	S	S	S	S	nil	B1
7	TL-20-W18	S	S	S	S	S	S	S	S	nil	A
8	TL-27-W18	S	S	S	S	S	S	S	S	nil	B1
9	TL-14-W18	S	S	S	S	S	S	R	S	nil	B1
10	TL-16-W18	S	S	S	S	S	S	S	S	nil	B1
11	TL-24-W18	S	S	S	S	S	S	S	S	nil	B1
12	TL-1-W18	S	S	S	S	S	S	S	S	nil	A
13	TL-25-W18	S	S	S	S	S	S	S	S	nil	unknown
14	TL-5-W18	S	S	S	S	S	S	S	S	nil	B1
15	TL-2-W18	S	S	S	S	S	S	S	S	nil	A
16	TL-18-W18	S	S	S	S	S	S	S	S	nil	B1
17	TL-50-W18	S	S	S	S	S	S	S	S	nil	unknown
18	TL-49-W18	S	S	S	S	S	S	S	S	nil	B1
19	TL-19-W18	S	S	S	S	S	S	S	S	nil	B1
20	TL-21-W18	S	S	S	S	S	S	S	S	nil	B1
21	TL-32-W18	S	S	S	S	S	S	S	S	nil	B1
22	TL-39-W18	S	S	S	S	S	S	S	S	nil	B1
23	TL-40-W18	S	S	S	S	S	S	S	S	nil	B1
24	TL-38-W18	S	S	S	S	S	S	S	S	nil	B1
25	TL-42-W18	S	S	S	S	S	S	S	S	nil	B1

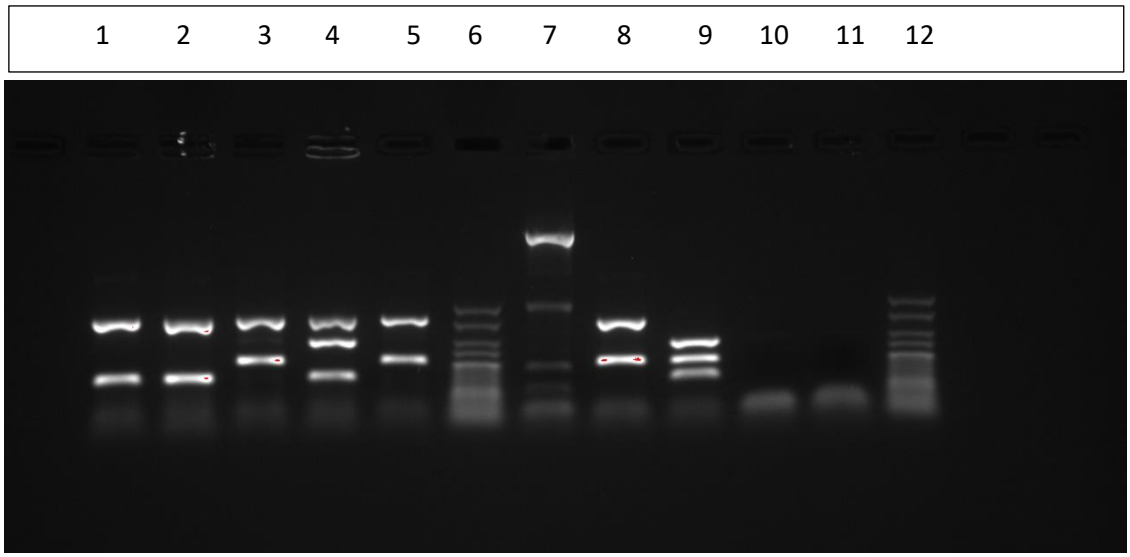
26	TL-43-W18	S	S	S	S	S	S	S	S	nil	B1
27	TL-44-W18	S	S	S	S	S	S	S	S	nil	B1
28	TL-41-W18	S	S	S	S	S	S	S	S	nil	B1
29	TL-33-W18	S	S	S	S	S	S	S	S	nil	B1
30	TL-31-W18	S	S	S	S	S	S	S	S	nil	B1
31	TL-30-W18	S	S	S	S	S	S	S	S	nil	B1
32	TL-29-W18	S	S	S	S	S	S	S	S	nil	B1
33	TL-35-W18	S	S	S	S	S	S	S	R	nil	B1
34	TL-13-W18	S	S	S	S	S	S	S	S	nil	A
35	TL-45-W18	S	S	S	S	S	S	S	S	nil	B1
36	TL-48-W18	S	S	S	S	S	S	S	S	nil	B1
37	TL-47-W18	S	S	S	S	S	S	S	S	nil	B1
38	TL-46-W18	S	S	S	S	S	S	S	S	nil	B1
39	TL-9-W18	S	S	S	S	S	S	S	S	nil	B1
40	TL-22-W18	S	S	S	S	S	S	S	S	nil	B1
41	TL-15-W18	S	S	S	S	S	S	S	S	nil	B1
42	TL-17-W18	S	S	S	S	S	S	S	S	nil	B1
43	TL-4-W18	S	S	S	S	S	S	S	S	nil	B1
44	TL-7-W18	S	S	S	S	S	S	S	S	nil	B1
45	TL-26-W18	S	S	S	S	S	S	S	S	nil	cladeIII +IV+V
46	TL-28-W18	S	S	S	S	S	S	R	S	nil	A
47	TL-36-W18	S	S	S	S	S	S	R	S	nil	unknown

Isolates in bold showed ≤10 mm inhibition zone to FOX30

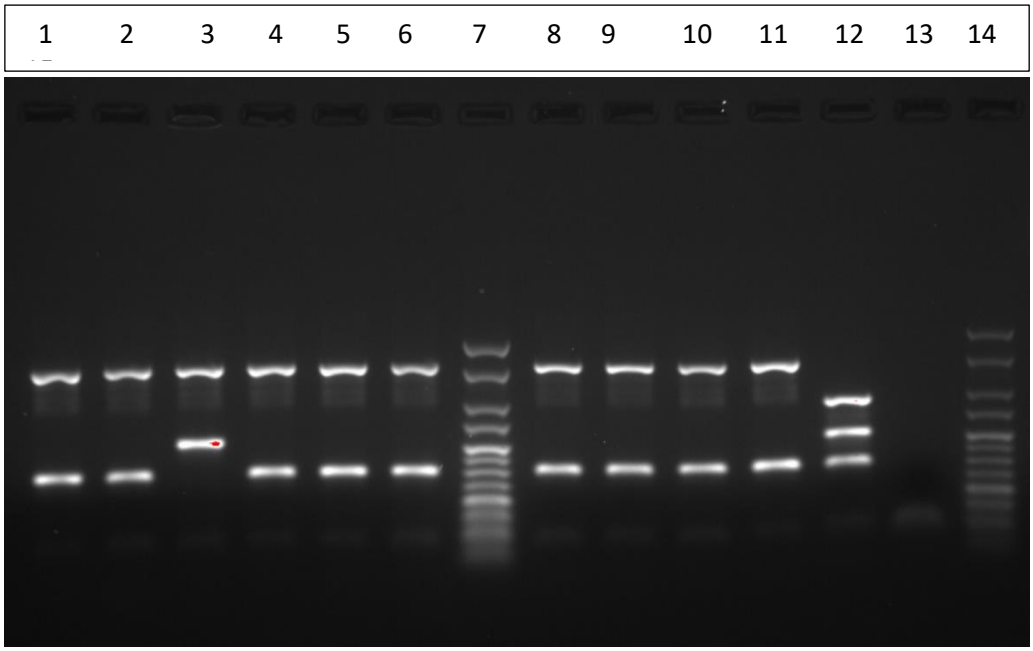
Appendix B

Phylogenetic typing of dairy farm E. coli isolates

Appendix D 1 Phylogenetic typing of DfSEC



S.No		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	TL-30-S18	+	-	-	+	B1
2	TL-34-S18	+	-	-	+	B1
3	CW-19-S18	+	-	+	-	A/C
4	CW-33-S18	+	+	-	+	D/E
5	CW-16-A18	+	-	+	-	A/C
6	Hyper ladder V					
7	CW-15-A18	-	-	-	-	not detected
8	*E. coli* NCTC 13351	+	-	+	-	A/C
9	*E. coli* ATCC25922	-	+	+	+	B2
10	Non-Template Control					
11	Non-Template Control					
12	Hyper ladder V					



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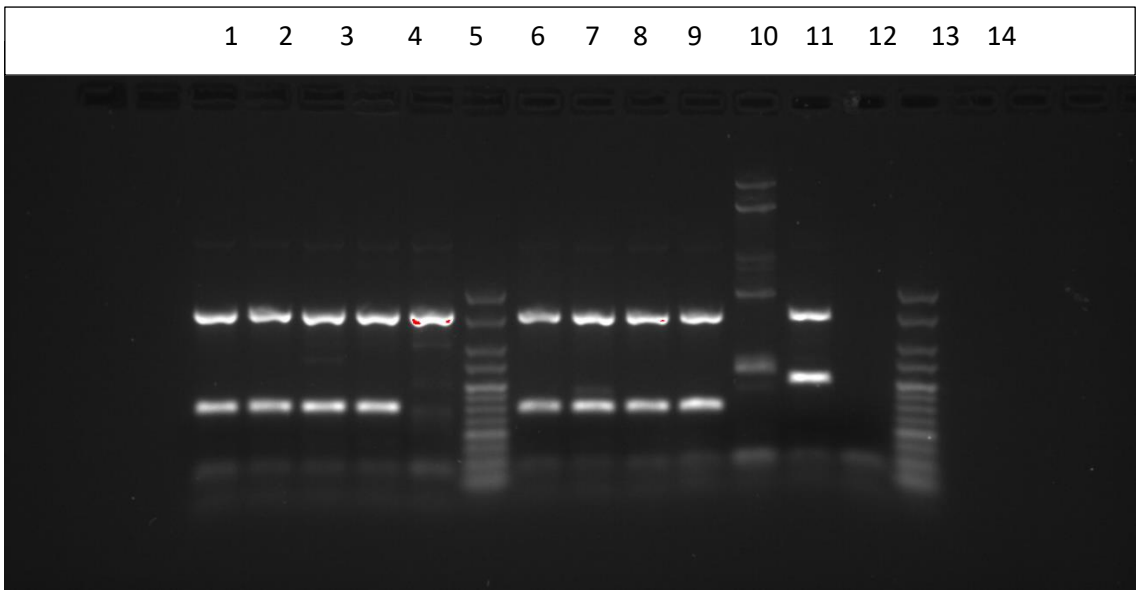
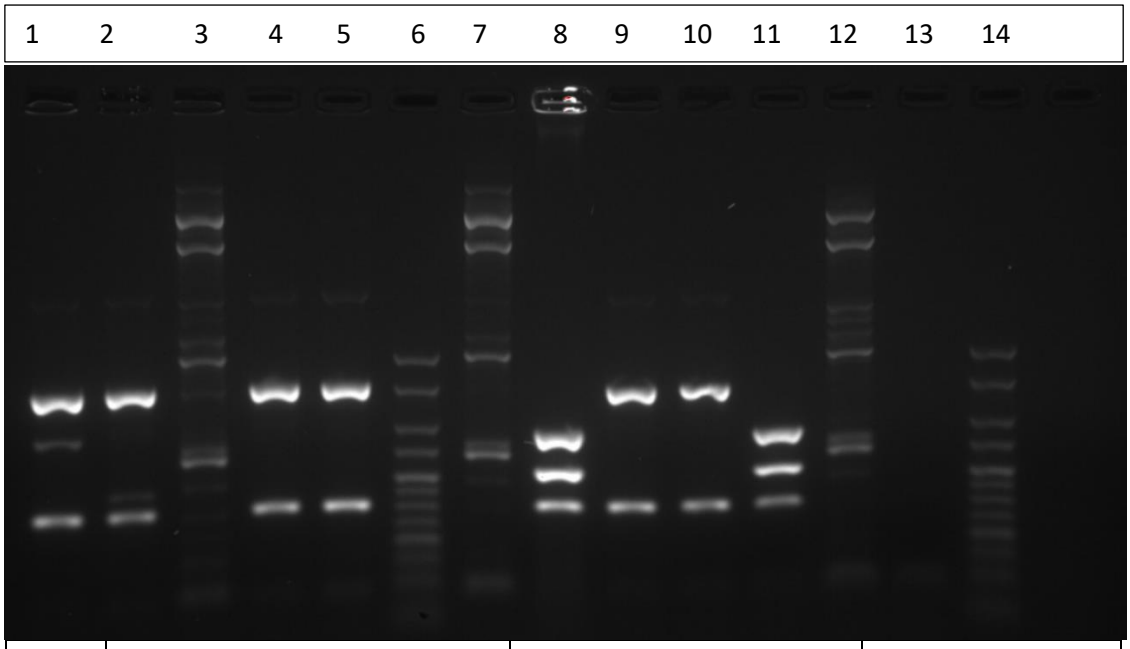
S.No		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	MRD -34-S17	+	-	-	+	B1
2	MRD-37-S17	+	-	-	+	B1
3	MRD-29-S17	+	-	+	-	A/C
4	MRD-38-S17	+	-	-	+	B1
5	MRD-19-S17	+	-	-	+	B1
6	MRD-30-S17	+	-	-	+	B1
7	Hyper ladder V					
8	MRD-24-S17	+	-	-	+	B1
9	MRD-35-S17	+	-	-	+	B1
10	MRD-18-S17	+	-	-	+	B1
11	MRD-27-S17	+	-	-	+	B1
12	*E. coli* ATCC25922	-	+	+	+	B2
13	Non-Template Control					
14	Hyper ladder V					

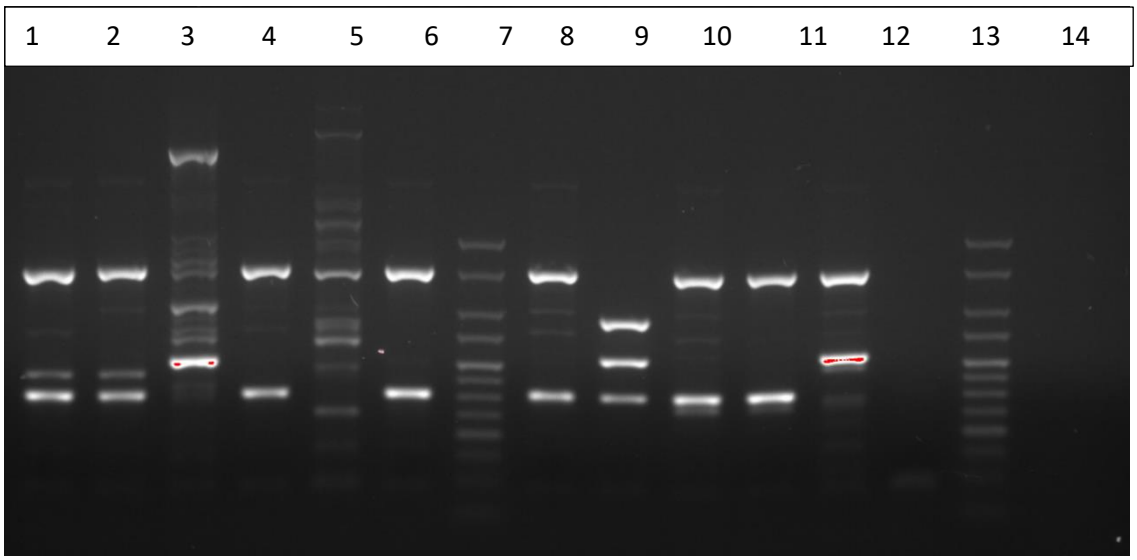


lincoln 2019-01-29_16h06m20s

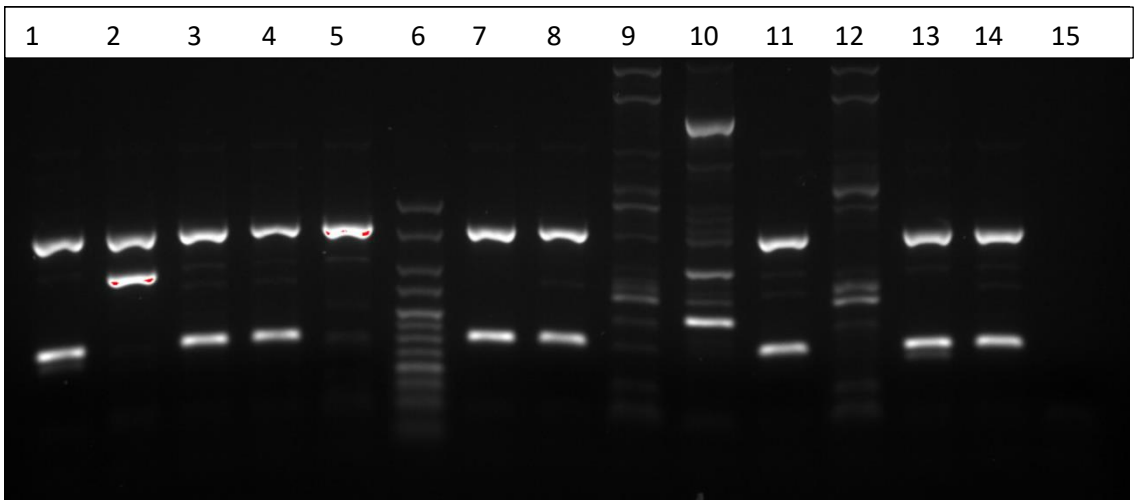
S.No		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	MRD-33-S17	+	-	-	+	B1
2	MRD-46-S17	+	-	-	+	B1
3	MRD-48-S17	+	-	-	+	B1
4	MRD-50-S17	+	-	-	+	B1
5	MRD-16-S17	+	-	-	-	A
6	Hyper ladder V					
7	MRD-13-S17	+	-	-	+	B1
8	MRD-14-S17	+	-	-	+	B1
9	MRD-11-S17	+	-	-	+	B1
10	MRD-33-S17	+	-	-	+	B1
11	MRD-9-S17	-	-	-	-	not detected
12	*E. coli* NCTC 13351	+	-	+	-	A/C
13	Non-template Control	-	-	-	-	
14	Hyper ladder V					



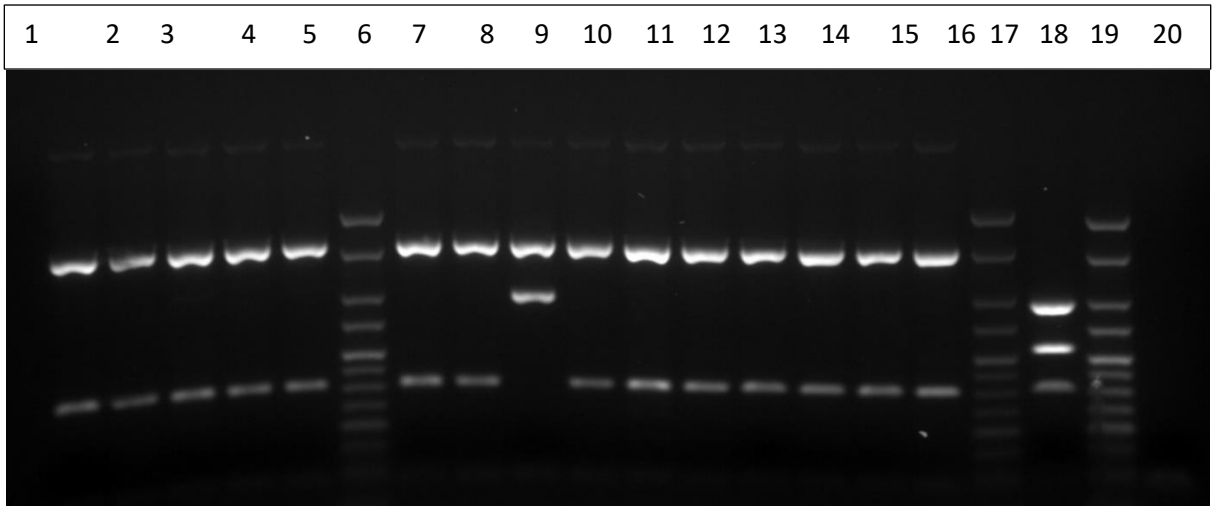
S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-1-S17	+	+	-	+	D/E
2	MRD-17-S17	+	-	-	+	B1
3	MRD-38-S17	-	-	-	-	not detected
4	MRD-15-S17	+	-	-	+	B1
5	MRD-49-S17	+	-	-	+	B1
6	Hyper ladder v (Marker)					
7	MRD-6-S17	-	-	-	-	not detected
8	MRD-40-S17	-	+	+	+	B2
9	MRD-44-S17	+	-	-	+	B1
10	MRD-2-S17	+	-	-	+	B1
11	ATCC25922 Positive control	-	+	+	+	B2
12	MRD-9-S17	-	-	-	-	not detected
13	NTC (Non-template control)	-	-	-	-	
14	Hyper ladder v (Marker)					



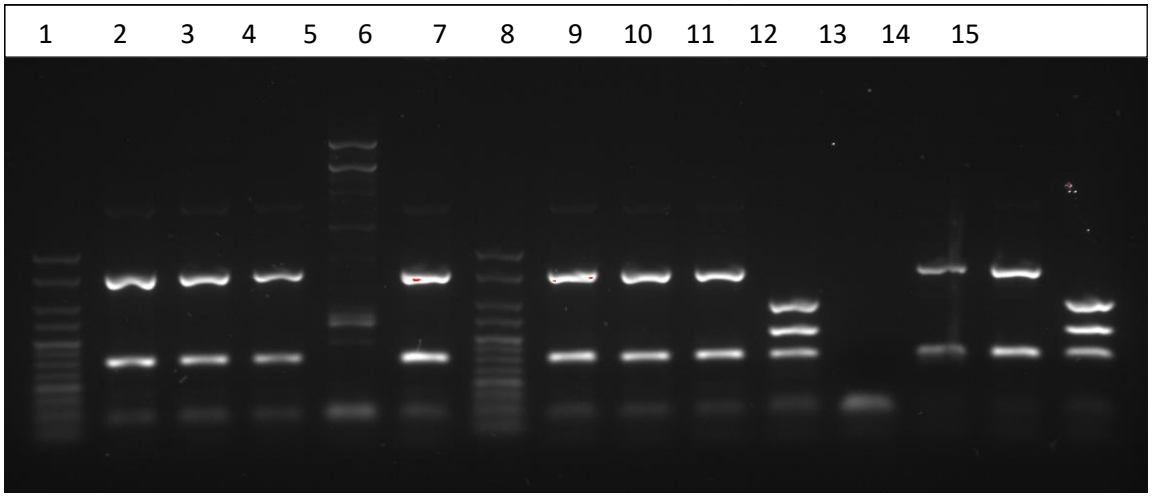
S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	TL-31-A18	+	-	-	+	B1
2	TL-29-A18	+	-	-	+	B1
3	TL-22-A18	-	-	-	-	not detected
4	TL-15-A18	+	-	-	+	B1
5	TL-10-A18	+	-	-	-	A
6	TL-47-A18	+	-	-	+	B1
7	Hyper ladder v (Marker)					
8	PF-47-A18	+	-	-	+	B1
9	ATCC25922 Positive control	-	+	+	+	B2
10	*E. coli* test 9 sample	+	-	-	+	B1
11	*E. coli* test 10 sample	+	-	-	+	B1
12	*E. coli* test 11 sample	+	-	+	-	A/C
13	NTC (Non-template control)					
14	Hyper ladder v (Marker)					



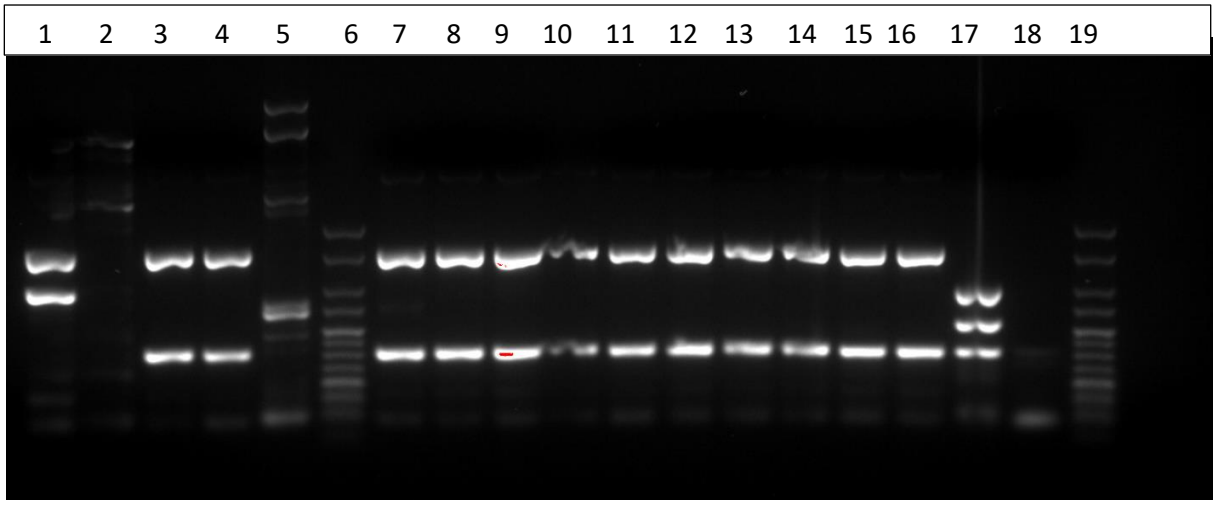
S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-49-S18	+	-	-	+	B1
2	PF-48-S18	+	+	-	-	D/E
3	PF-51-S18	+	-	-	+	B1
4	PF-52-S18	+	-	-	+	B1
5	PF-55-S18	+	-	-	-	A
6	Hyper ladder v (Marker)					
7	PF-40-S18	+	-	-	+	B1
8	PF-46-S18	+	-	-	+	B1
9	TL-2-S18	-	-	-	-	Not detected
10	TL-27-A18	-	-	-	-	Not detected
11	PF-39-S18	+	-	-	+	B1
12	PF-35-S18	-	-	-	-	Not detected
13	PF-34-S18	+	-	-	+	B1
14	PF-42-S18	+	-	-	+	B1
15	NTC (Non-template control)					



S.No		Quadruplex genotype				Phylogroup
Lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-33-S17	+	-	-	+	B1
2	CW-4-S17	+	-	-	+	B1
3	CW-9-S17	+	-	-	+	B1
4	CW-33-S17	+	-	-	+	B1
5	CW-9-S17	+	-	-	+	B1
6	Hyper ladder v (Marker)					
7	CW-20-S17	+	-	-	+	B1
8	CW-17-S17	+	-	-	+	B1
9	CW-39-S17	+	+	-	-	D+E
10	CW-35-S17	+	-	-	+	B1
11	CW-5-S17	+	-	-	+	B1
12	CW-18-S17	+	-	-	+	B1
13	MRD-50-S17	+	-	-	+	B1
14	MRD-51-S17	+	-	-	+	B1
15	MRD-42-S17	+	-	-	+	B1
16	MRD-40-S17	+	-	-	+	B1
17	Hyper ladder v (Marker)	-	-	-	-	
18	PTC (Positive Template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					
20	NTC (Non-template control	-	-	-	-	



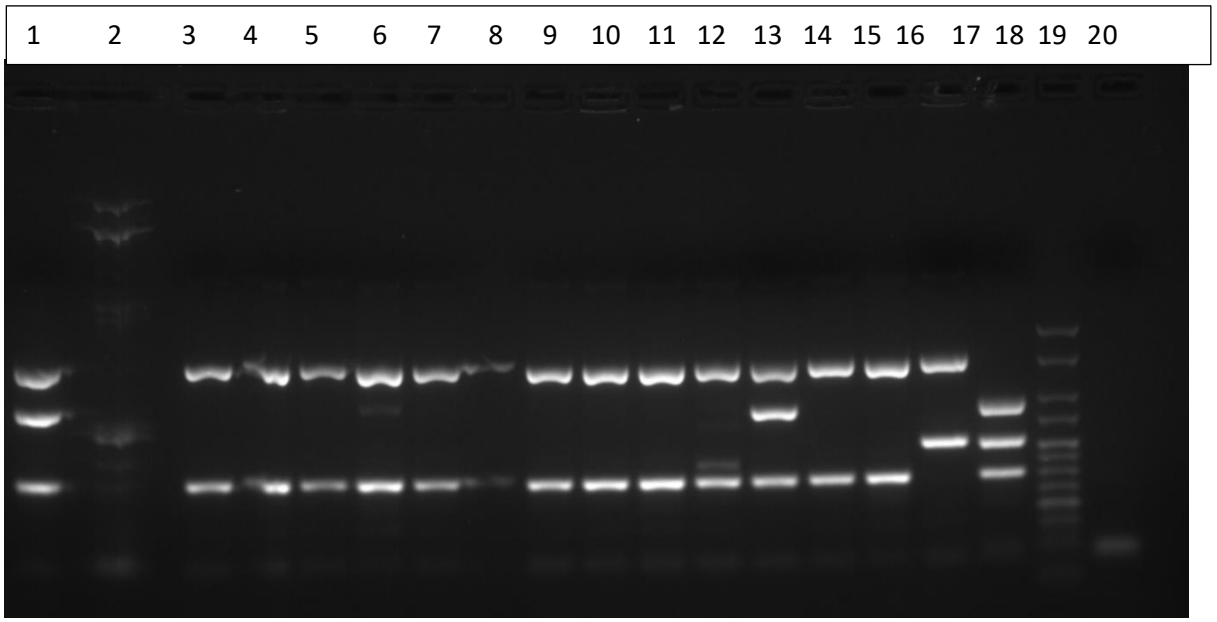
S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper ladder v (Marker)					
2	CW-16-S17	+	-	-	+	B1
3	MRD-4-S17	+	-	-	+	B1
4	CW-86-S17	+	-	-	+	B1
5	CW-30-S17					Not detected
6	CW-46-S17	+	-	-	+	B1
7	Hyper ladder v (Marker)					
8	CW-60-S17	+	-	-	+	B1
9	CW-1-S17	+	-	-	+	B1
10	CW-14-S17	+	-	-	+	B1
11	PTC (Positive template control) ATCC	-	+	+	+	B2
12	NTC (Non-template control	+	-	-	+	B1
13	PF-25-S18	+	-	-	+	B1
14	PF-36-S18	+	-	-	+	B1
15	PTC (Positive template control) ATCC	-	+	+	+	B2



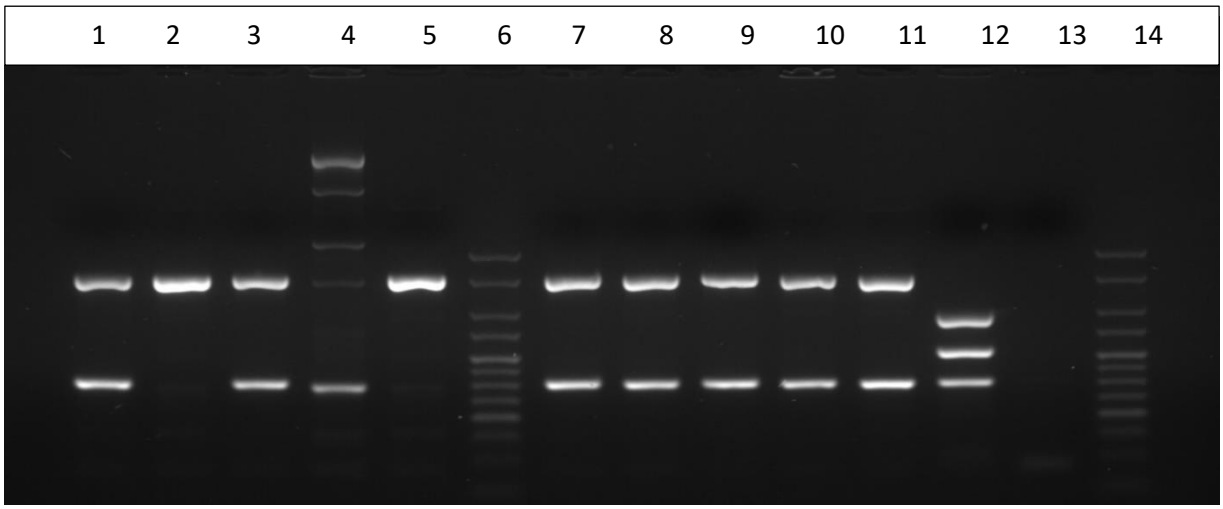
lincoln 2019-02-15_16h46m23s

Lane order and Quadruplex genotype

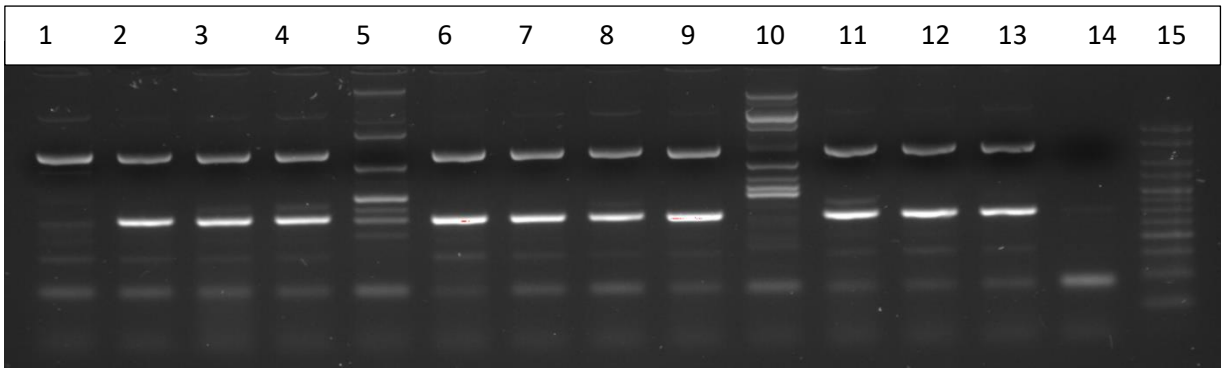
S.No		**Quadruplex genotype**				**Phylogroup**
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-38-S17	+	+	-	-	D/E
2	PF-56-S18					Not detected
3	CW-37-S17	+	-	-	+	B1
4	CW-36-S17	+	-	-	+	B1
5	CW-28-S17					Not detected
6	Hyper ladder v (Marker)					
7	CW-23-S17	+	-	-	+	B1
8	CW-48-S17	+	-	-	+	B1
9	CW-31-S17	+	-	-	+	B1
10	CW-35-S17	+	-	-	+	B1
11	CW-14-S17	+	-	-	+	B1
12	CW-39-S17	+	-	-	+	B1
13	CW-47-S17	+	-	-	+	B1
14	CW-42-S17	+	-	-	+	B1
15	CW-26-S17	+	-	-	+	B1
16	CW-38-S17	+	-	-	+	B1
17	PTC (Positive Template control) ATCC	-	+	+	+	B2
18	NTC (Non-template control	-	-	-	-	-
19	Hyper ladder v (Marker)					



S.No		Quadruplex genotype				Phylogroup
Lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	CW-35-W18	+	+	-	+	D/E
2	CW-25-S17					Not detected
3	CW-34-S17	+	-	-	+	B1
4	CW-50-S17	+	-	-	+	B1
5	CW-23-S17	+	-	-	+	B1
6	CW-47-S17	+	-	-	+	B1
7	CW-7-S17	+	-	-	+	B1
8						
9	CW-11-S17	+	-	-	+	B1
10	CW-34-S17	+	-	-	+	B1
11	CW-8-S17	+	-	-	+	B1
12	CW-40-S17	+	-	-	+	B1
13	CW-49-S17	+	+	-	+	D/E
14	CW-21-S17	+	-	-	+	B1
15	CW-6-S17	+	-	-	+	B1
16	CW-10-S17	+	-	+	-	A/C
17	PTC (Positive Template control) ATCC	-	+	+	+	B2
18	Hyper ladder v (Marker)	-	-	-	-	-
19	NTC (Non-template control)					



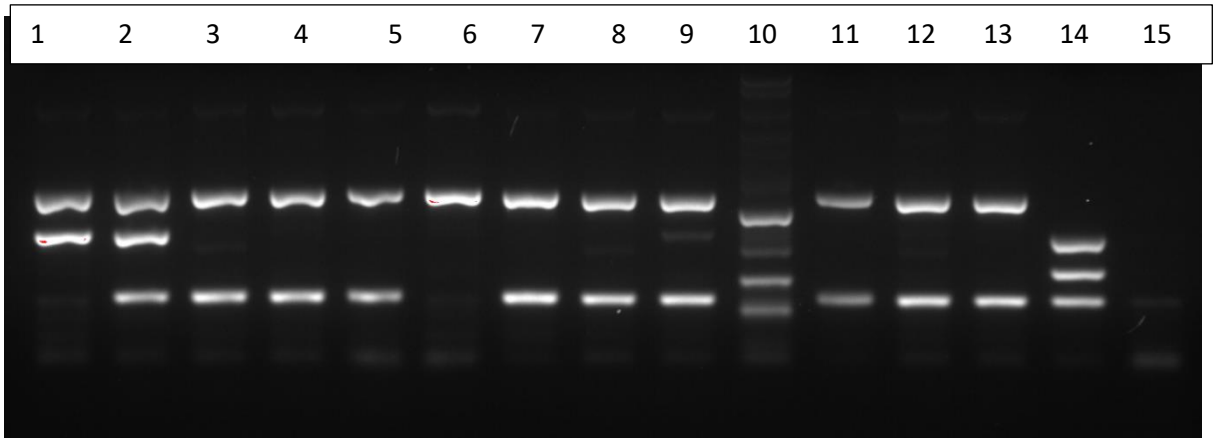
S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-16-W18	+	-	-	+	B1
2	MRD-19-W18	+	-	-	-	A
3	CW-15-S17	+	-	-	+	B1
4	MRD-6-W18	-	-	-	+	**Unknown perform MLST**
5	MRD-18-W18	+	-	-	-	A
6	Hyper ladder v (Marker)					
7	CW-52-S17	+	-	-	+	B1
8	MRD-15-W18	+	-	-	+	B1
9	MRD-36-W18	+	-	-	+	B1
10	MRD-1-W18	+	-	-	+	B1
11	CW-45-S17	+	-	-	+	B1
12	PTC (Positive template control) ATCC	-	+	+	+	B2
13	NTC (Non-template control	+	-	-	+	B1
14	Hyper ladder v (Marker)	-	+	+	+	B2



lincoln 2019-02-27_14h43m23s

Lane order and Quadruplex genotype

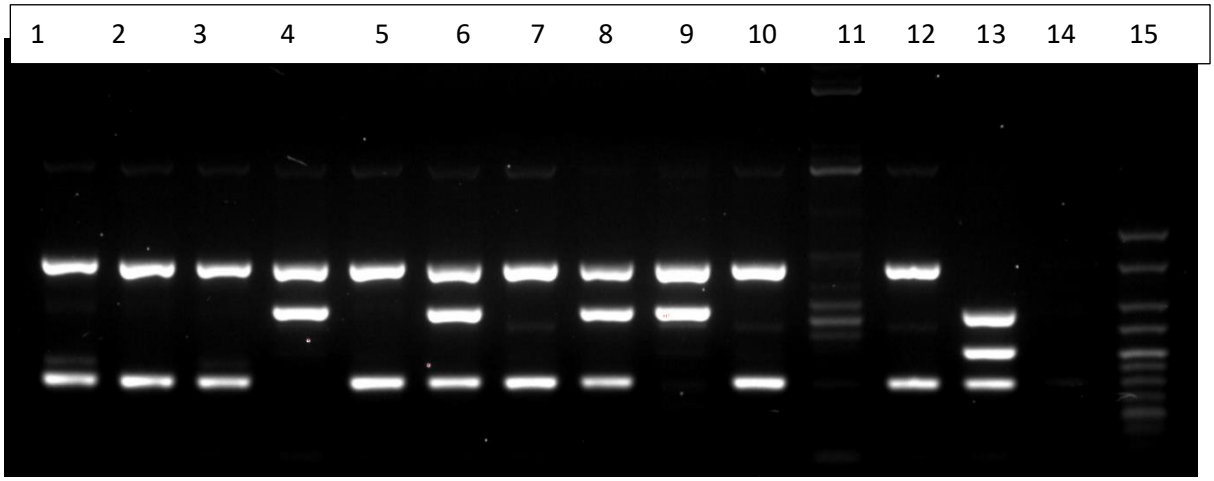
S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	MRD-61-A18	+	-	-	-	A
2	MRD-43-A18	+	-	-	+	B1
3	MRD-39-W18	+	-	-	+	B1
4	MRD-50-W18	+	-	-	+	B1
5	MRD-41-W18	-	-	+	+	UNKNOWN
6	MRD-49-W18	+	-	-	+	B1
7	MRD-55-W18	+	-	-	+	B1
8	MRD-48-W18	+	-	-	+	B1
9	MRD-56-A18	+	-	-	+	B1
10	MRD-57-A18					Not detected
11	MRD-62-A18	+	-	-	+	B1
12	MRD-63-A18	+	-	-	+	B1
13	MRD-64-A18	+	-	-	+	B1
14	NTC (Non-template control)					
17	Hyper ladder v (Marker)					



lincoln 2019-03-01_16h39m50s

Lane order and Quadruplex genotype

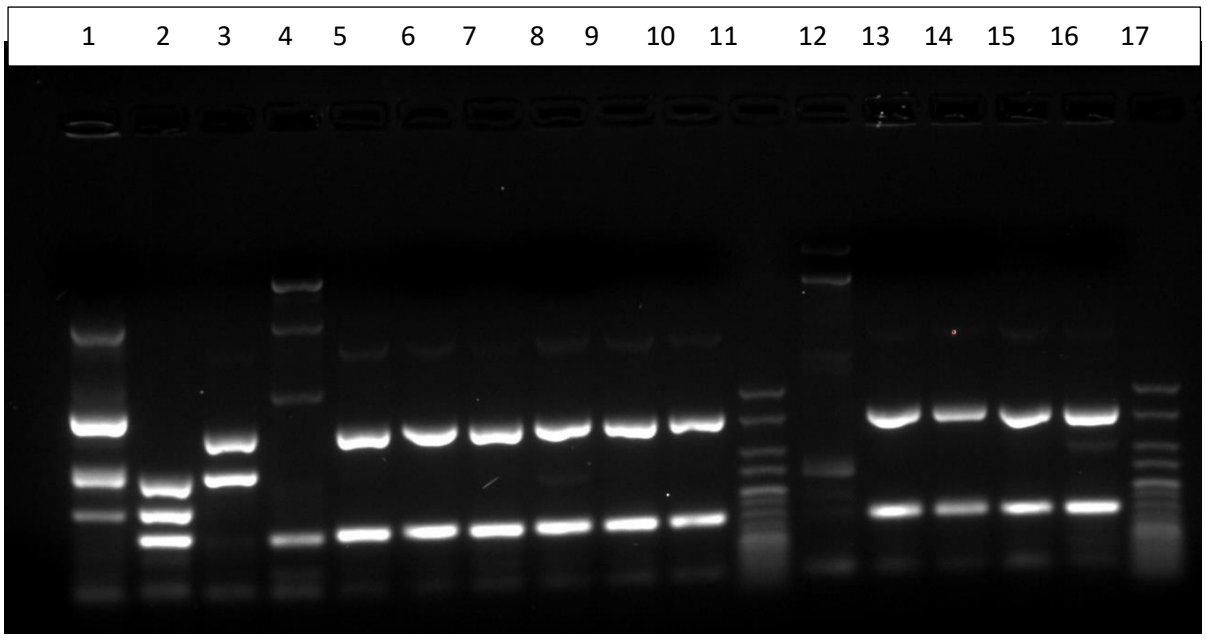
S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	*chu A*	*yja A*	*TspE4C2*	
1	MRD-25-W18	+	+	-	-	D/E
2	MRD-55-A18	+	+	-	+	D/E
3	MRD-50-A18	+	-	-	+	B1
4	MRD-44-A18	+	-	-	+	B1
5	MRD-41-A18	+	-	-	+	B1
6	MRD-52-A18	+	-	-	-	A
7	MRD-59-A18	+	-	-	+	B1
8	MRD-51-A18	+	-	-	+	B1
9	MRD-53-A18	+	-	-	+	B1
10	MRD-42-A18					Not detected
11	MRD-33-A18	+	-	-	+	B1
12	MRD-38-A18	+	-	-	+	B1
13	MRD-40-A18	+	-	-	+	B1
14	PTC (Positive template control) ATCC	-	+	+	+	B2
15	NTC (Non-template control)					



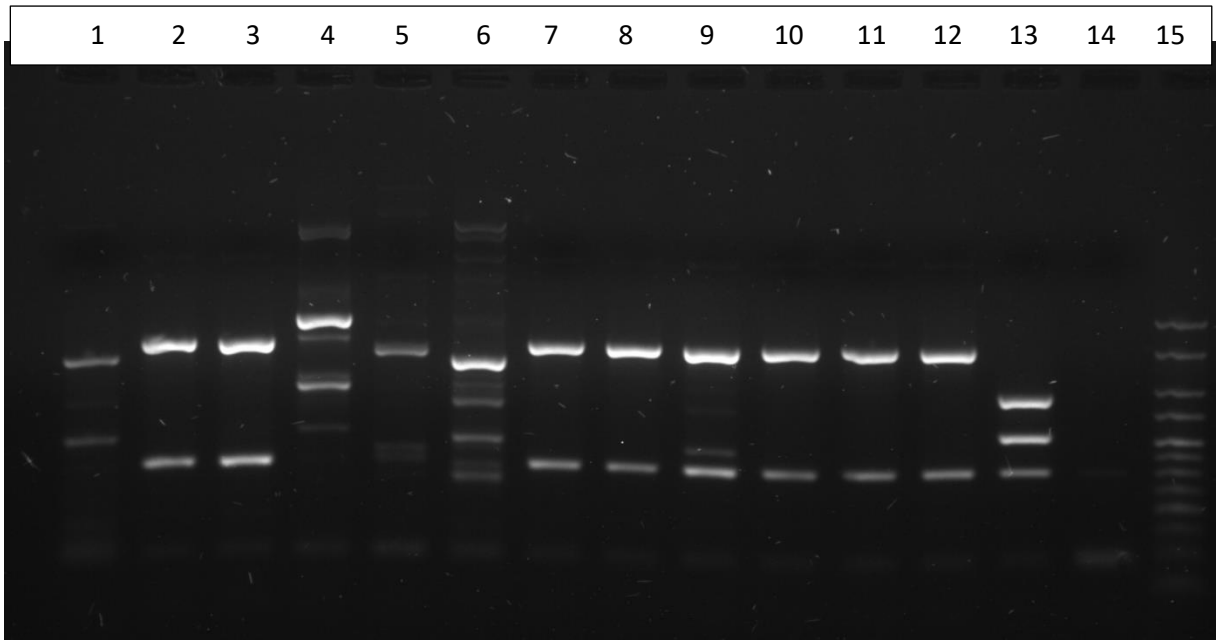
lincoln 2019-03-01_16h07m46s

Lane order and Quadruplex genotype

S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	MRD-38-W18	+	-	-	+	B1
2	MRD-44-W18	+	-	-	+	B1
3	MRD-43-W18	+	-	-	+	B1
4	MRD-47-W18	+	+	-	-	D/E
5	MRD-42-W18	+	-	-	+	B1
6	MRD-46-W18	+	+	-	+	D/E
7	MRD-30-W18	+	-	-	+	B1
8	MRD-31-W18	+	+	-	+	D/E
9	MRD-37-A18	+	+	-	-	D/E
10	MRD-47-A18	+	-	-	+	B1
11	MRD-27-W18	-	-	-	-	Unknown*
12	MRD-48-W18	+	-	-	+	B1
13	PTC (Positive template control) ATCC	-	+	+	+	B2
14	NTC (Non-template control)					
15	Hyper ladder v (Marker)					



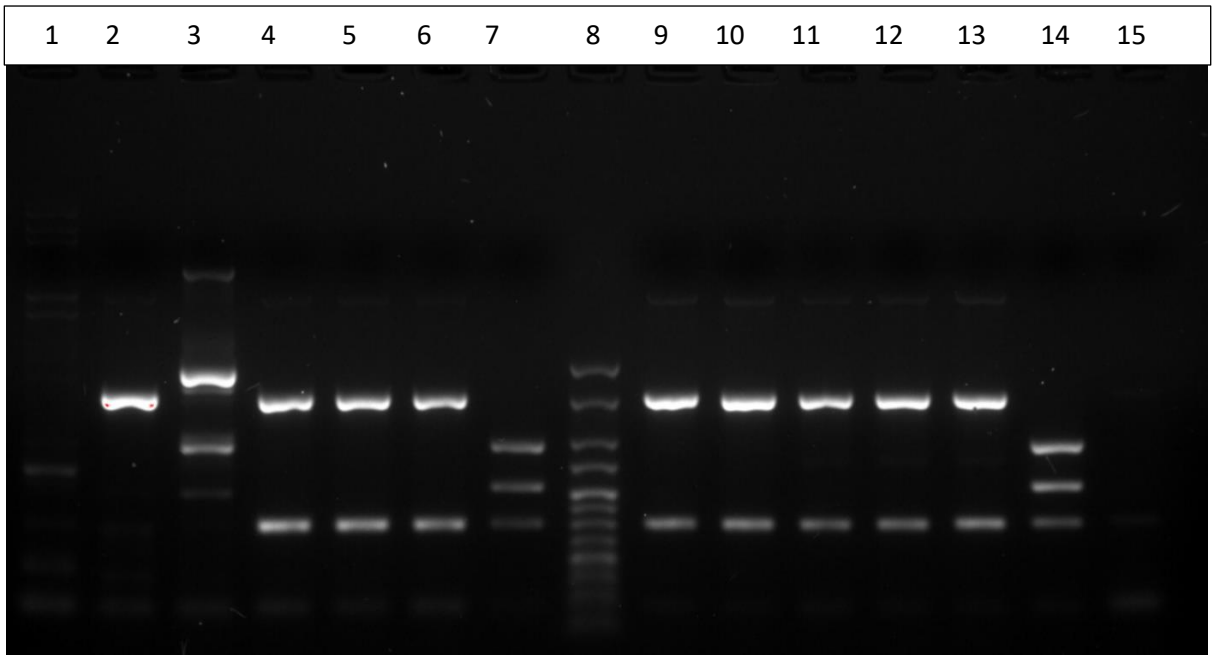
S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	*chu A*	*yja A*	*TspE4C2*	
1	MRD-49-A18	+	+	+	-	E or clade 1
2	PTC (Positive template control) ATCC	-	+	+	+	B2
3	MRD-26-W18	+	+	-	-	D/E
4	MRD-20-W18	-	-	-	+	**Unknown perform MLST**
5	MRD-46-A18	+	-	-	+	B1
6	MRD-48-A18	+	-	-	+	B1
7	MRD-45-A18	+	-	-	+	B1
8	MRD-29-W18	+	-	-	+	B1
9	MRD-37-W18	+	-	-	+	B1
10	MRD-35-W18	+	-	-	+	B1
11	Hyper ladder v (Marker)					
12	CW-25-S17					Not detected
13	CW-34-S17	+	-	-	+	B1
14	CW-50-S17	+	-	-	+	B1
15	CW-23-S17	+	-	-	+	B1
16	CW-7-S17	+	-	-	+	B1
17	Hyper ladder v (Marker)					



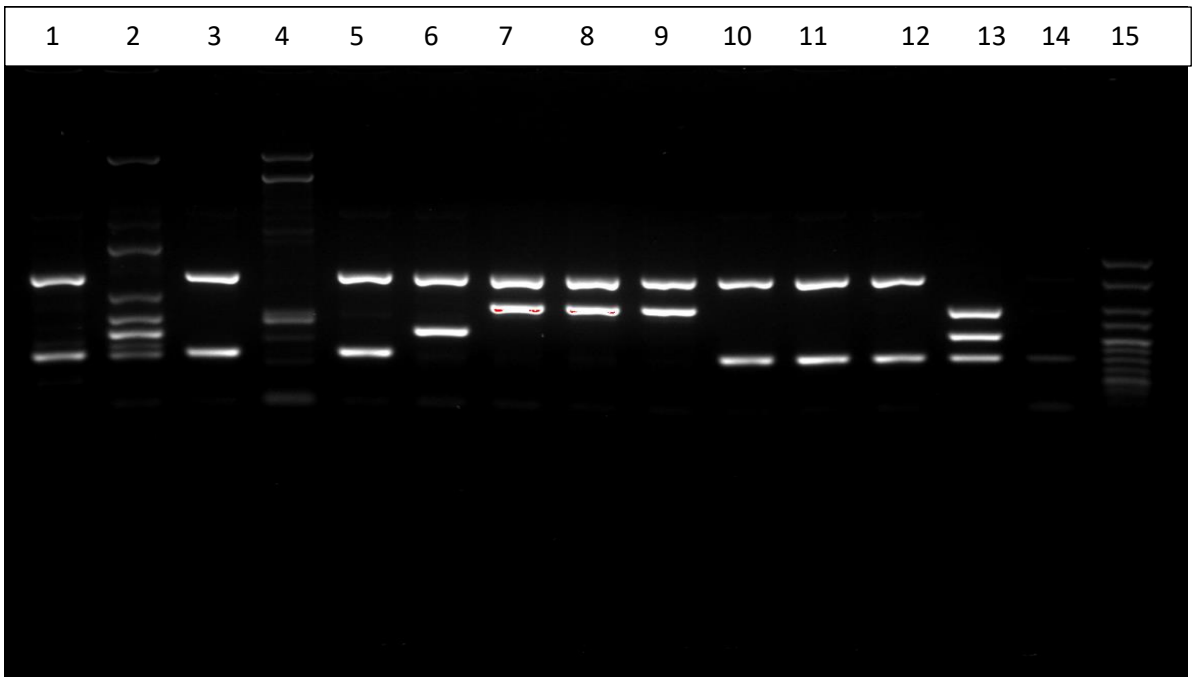
lincoln 2019-03-06_15h10m53s

Lane order and Quadruplex genotype

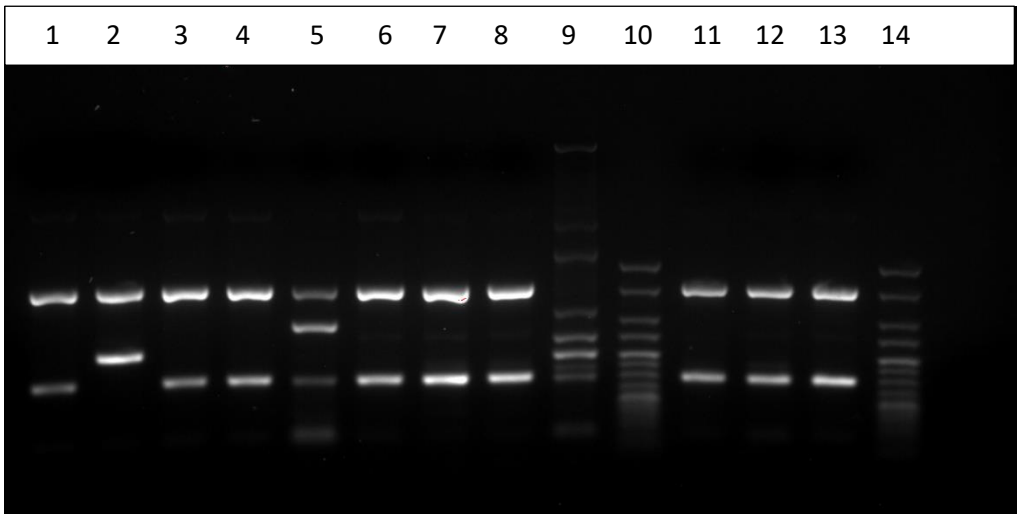
S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	*chu A*	*yja A*	*TspE4C2*	
1	MRD-9-A18	-	-	-	-	Not detected
2	MRD-1-A18	+	-	-	+	B1
3	MRD-10-A18	+	-	-	+	B1
4	MRD-35-A18	476 bp	-	-	-	Clade III, IV or V
5	MRD-2-A18	+	-	-	-	A
6	MRD-5-A18	-	-	-	-	Not detected
7	MRD-7-A18	+	-	-	+	B1
8	MRD-30-A18	+	-	-	+	B1
9	MRD-31-A18	+	-	-	+	B1
10	MRD-6-A18	+	-	-	+	B1
11	MRD-8-A18	+	-	-	+	B1
12	MRD-32-A18	+	-	-	+	B1
13	PTC (Positive template control) ATCC	-	+	+	+	B2
14	NTC (Non-template control)	-	-	-	-	
15	Hyper ladder v (Marker)					



S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	*chu A*	*yja A*	*TspE4C2*	
1	MRD-11-A18	-	-	-	-	Not detected
2	MRD-3-A18	+	-	-	-	A
3	MRD-35-A18	476 bp	-	-	-	**Clade III, IV or V**
4	MRD-18-A18	+	-	-	+	B1
5	MRD-16-A18	+	-	-	+	B1
6	MRD-17-A18	+	-	-	+	B1
7	PTC (Positive template control) ATCC	-	+	+	+	B2
8	Hyper ladder v (Marker)					
9	PF-56-A18	+	-	-	+	B1
10	PF-29-A18	+	-	-	+	B1
11	PF-34-A18	+	-	-	+	B1
12	PF-1-A18	+	-	-	+	B1
13	PF-3-A18	+	-	-	+	B1
14	PTC (Positive template control) ATCC	-	+	+	+	B2
15	NTC (Non-template control)					



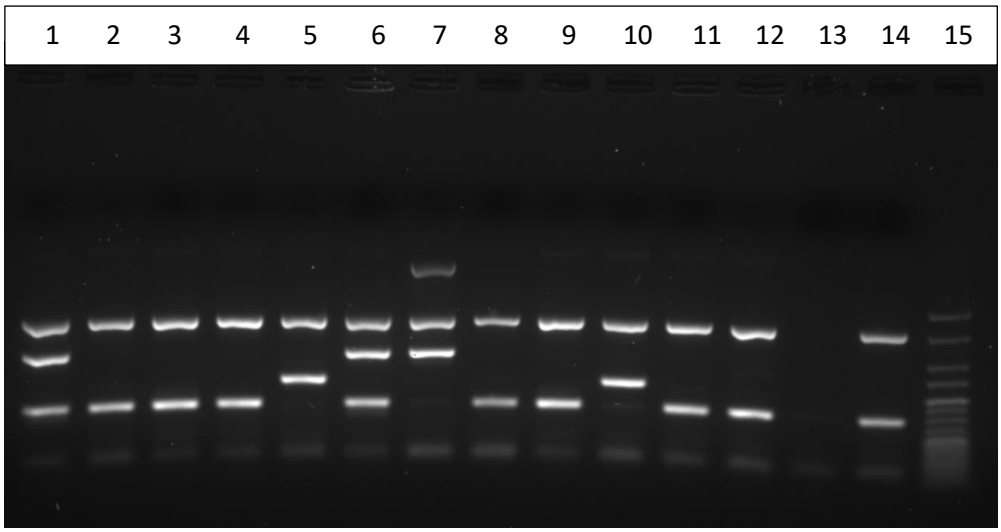
S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-28-A18	+	-	-	+	B1
2	PF-39-A18	-	-	-	-	Not detected
3	PF-27-A18	+	-	-	+	B1
4	PF-53-A18	-	-	-	-	Not detected
5	PF-8-A18	+	-	-	+	B1
6	PF-54-A18	+	-	+	-	A/C
7	PF-43-A18	+	+	-	-	D/E
8	PF-57-A18	+	+	-	-	D/E
9	PF-55-A18	+	+	-	-	D/E
10	PF-38-A18	+	-	-	+	B1
11	PF-47-A18	+	-	-	+	B1
12	PF-52-A18	+	-	-	+	B1
13	PTC (Positive template control) ATCC	-	+	+	+	B2
14	NTC (Non-template control)					
15	Hyper ladder v (Marker)					



lincoln 2019-03-07_17h41m14s

Lane order and Quadruplex genotype

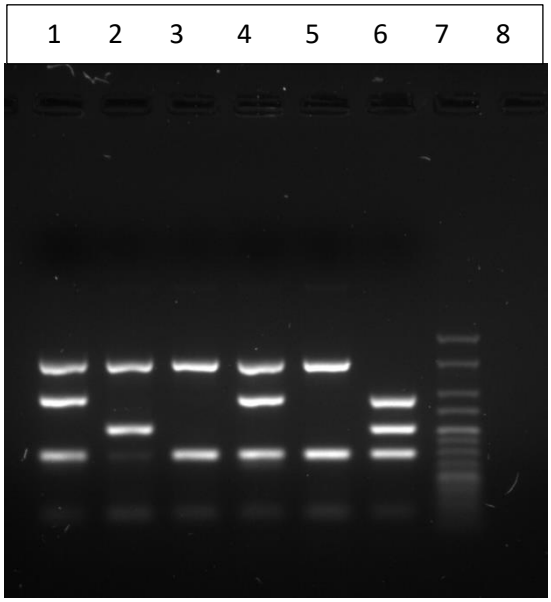
S.No		**Quadruplex genotype**				**Phylogroup**
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-49-A18	+	-	-	+	B1
2	PF-51-A18	+	-	+	-	A/C
3	PF-13-A18	+	-	-	+	B1
4	PF-2-A18	+	-	-	+	B1
5	PF-48-A18	+	+	-	+	D/E
6	PF-20-A18	+	-	-	+	B1
7	PF-32-A18	+	-	-	+	B1
8	PF-6-A18	+	-	-	+	B1
9	PF-17-A18	-	-	-	-	Not detected
10	Hyper ladder v (Marker)					
11	PF-33-A18	+	-	-	+	B1
12	PF-42-A18	+	-	-	+	B1
13	PF-26-A18	+	-	-	+	B1
14	Hyper ladder v (Marker)					



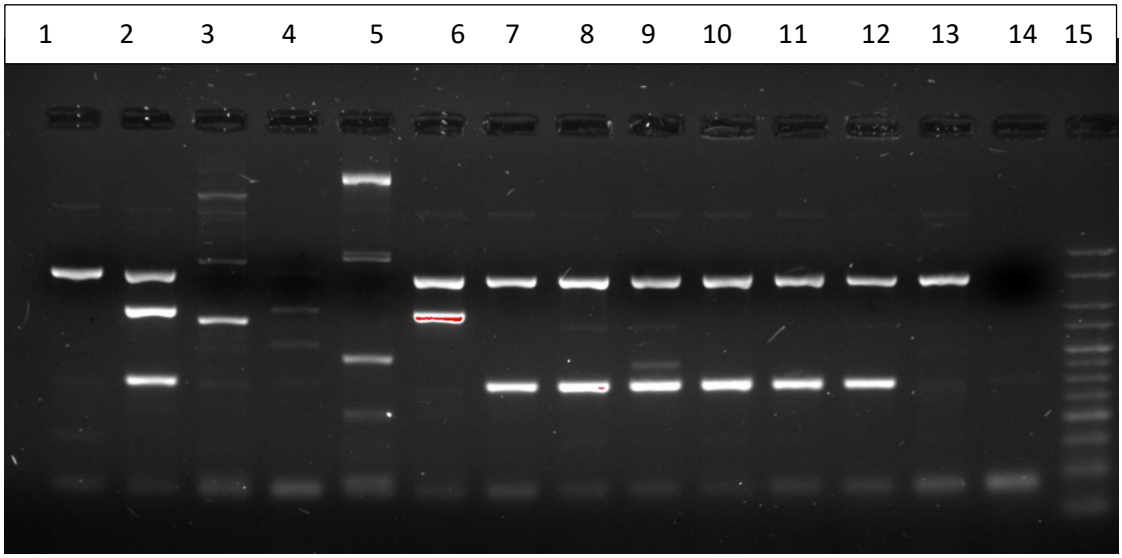
lincoln 2019-03-14_12h33m03s

Lane order and Quadruplex genotype

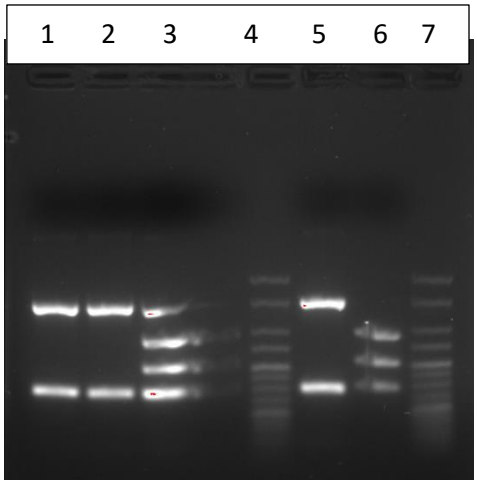
S.No		**Quadruplex genotype**				**Phylogroup**
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	CW-1-A18	+	+	-	+	D/E
2	CW-2-A18	+	-	-	+	B1
3	CW-10-A18	+	-	-	+	B1
4	PF-10-A18	+	-	-	+	B1
5	CW-41-A18	+	-	+	-	A/C
6	PF-12-A18	+	+	-	+	D/E
7	CW-14-A18	+	+	-	-	D/E
8	CW-3-A18	+	-	-	+	B1
9	CW-9-A18	+	-	-	+	B1
10	CW-31-A18	+	-	+	-	A/C
11	CW-43-A18	+	-	-	+	B1
12	PF-37-A18	+	-	-	+	B1
13	NTC (Non-template control)					
14	CW-6-A18	+	-	-	+	B1
15	Hyper ladder v (Marker)					

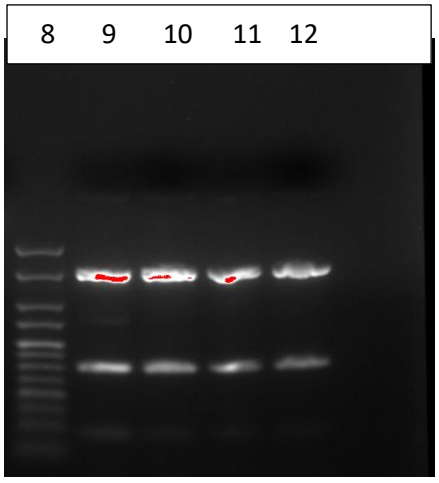


S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-40-A18	+	+	-	+	D/E
2	CW-43-A18	+	-	+	-	A/C
3	CW-87-S17	+	-	-	+	B1
4	CW-8-A18	+	+	-	+	D/E
5	CW-44-A18	+	-	-	+	B1
6	PTC (Positive template control) ATCC	-	+	+	+	B2
7	Hyper ladder v (Marker)					
8	NTC (Non-template control)					

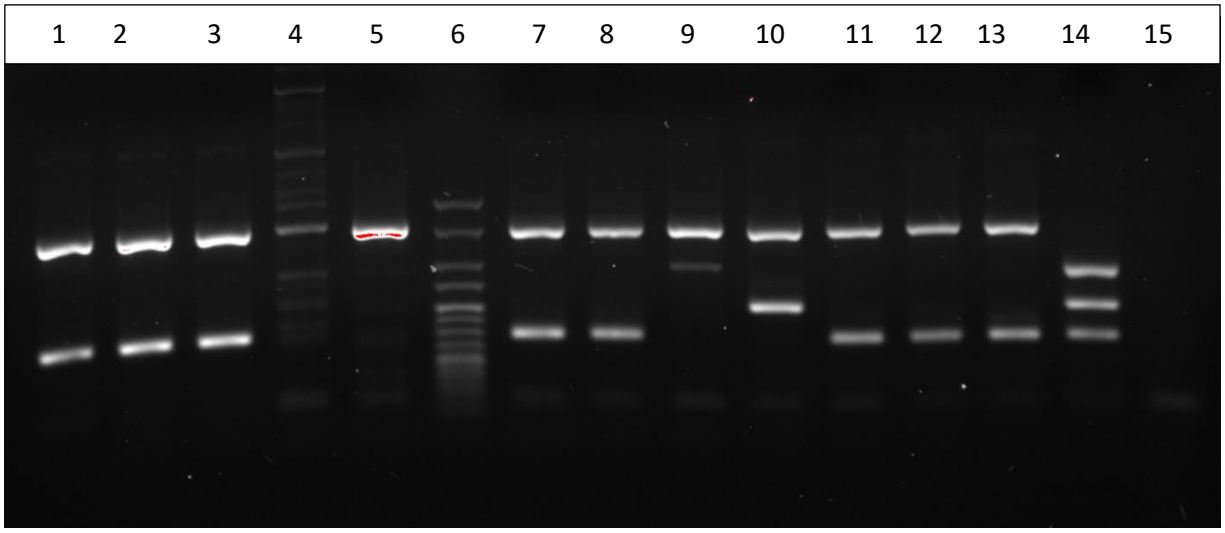


		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-13-W18	+	-	-	-	A
2	CW-36-A18	+	+	-	+	D/E
3	CW-20-A18					Not assignable
4	CW-35-A18					Not assignable
5	CW-27-A18					Not assignable
6	CW-23-A18	+	+	-	-	D/E
7	CW-48-A18	+	-	-	+	B1
8	PF-3-A18	+	-	-	+	B1
9	PF-7-A18	+	-	-	+	B1
10	MRD-9-W18	+	-	-	+	B1
11	CW-5-A18	+	-	-	+	B1
12	PF-4-A18	+	-	-	+	B1
13	MRD-11-W18	+	-	-	-	A
14	NTC (Non-template control)					
15	Hyper ladder v (Marker)					

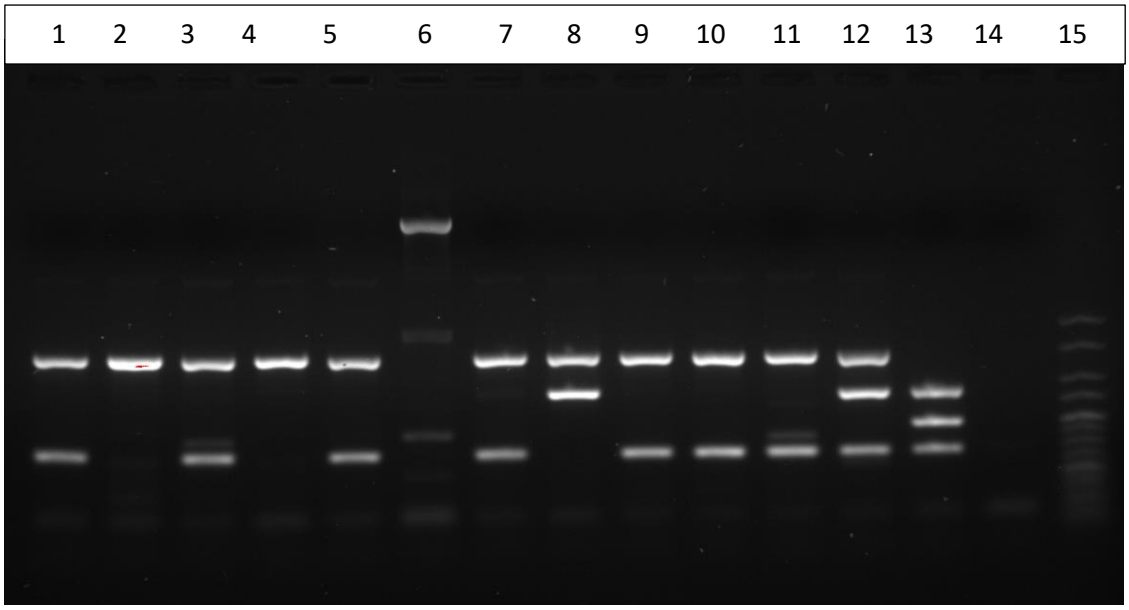




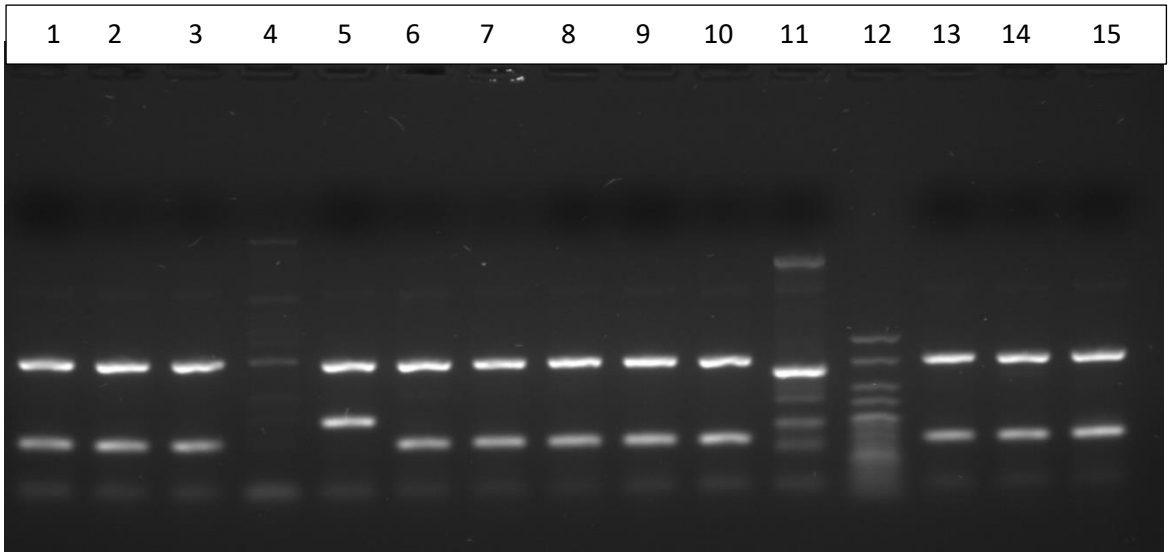
S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	CW-22-S17	+	-	-	+	B1
2	PF-38-A18	+	-	-	+	B1
3	CW-20-A18					
4	Hyper ladder v (Marker)					
5	CW-4-A18	+	-	-	+	B1
6	PTC (Positive template control) ATCC	-	+	+	+	B2
7	Hyper ladder v (Marker)					
8	Hyper ladder v (Marker)					
9	PF-8-A18	+	-	-	+	B1
10	PF-38-A18	+	-	-	+	B1
11	CW-4-A18	+	-	-	+	B1
12	CW-33-A18	+	-	-	+	B1



		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	CW-19-S17	+	-	-	+	B1
2	CW-50-A18	+	-	-	+	B1
3	CW-45-S17	+	-	-	+	B1
4	MRD-5-W18	+	-	-	-	A
5	MRD-7-W18	+	-	-	-	A
6	Hyper ladder v (Marker)					
7	MRD-14-S18	+	-	-	+	B1
8	MRD-15-S18	+	-	-	+	B1
9	TL-13-S18	+	-	-	-	A
10	MRD-25-S18	+	-	+	-	A/C
11	MRD-35-S18	+	-	-	+	B1
12	MRD-4-S18	+	-	-	+	B1
13	MRD-16-S18	+	-	-	+	B1
14	PTC (Positive template control) ATCC	-	+	+	+	B2
15	NTC (Non-template control)					



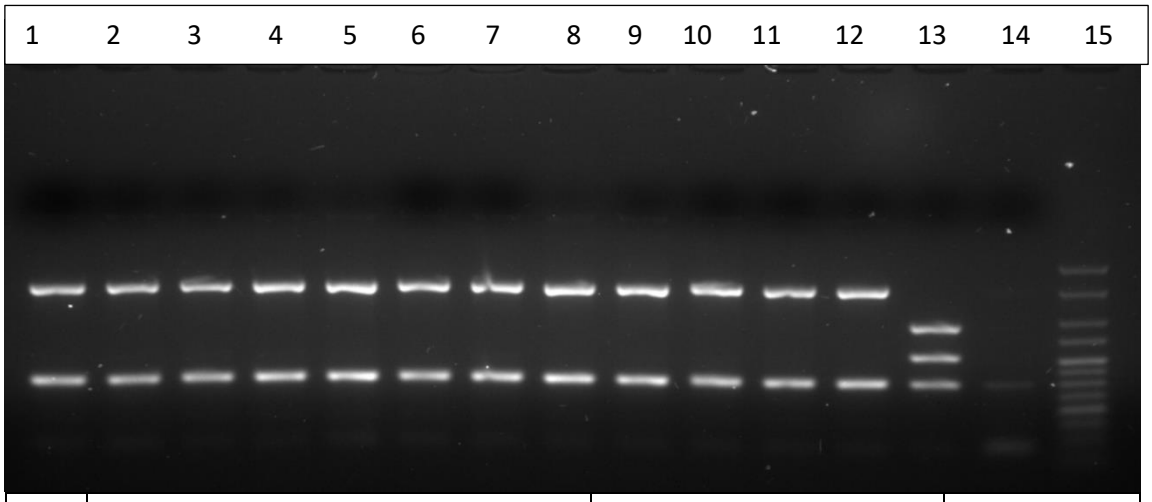
S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	CW-40-A18	+	-	-	+	B1
2	MRD-14-W18	+	-	-	-	A
3	CW-34-A18	+	-	-	+	B1
4	MRD-10-W18	+	-	-	-	A
5	CW-13-A18	+	-	-	+	B1
6	CW-46-A18					Not assignable
7	CW-32-A18	+	-	-	+	B1
8	CW-37-A18	+	+	-	-	D/E
9	CW-29-A18	+	-	-	+	B1
10	CW-38-W18	+	-	-	+	B1
11	CW-21-A18	+	-	-	+	B1
12	CW-18-A18	+	+	-	+	D/E
13	PTC (Positive template control) ATCC					
14	NTC (Non-template control)					
15	Hyper ladder v (Marker)					



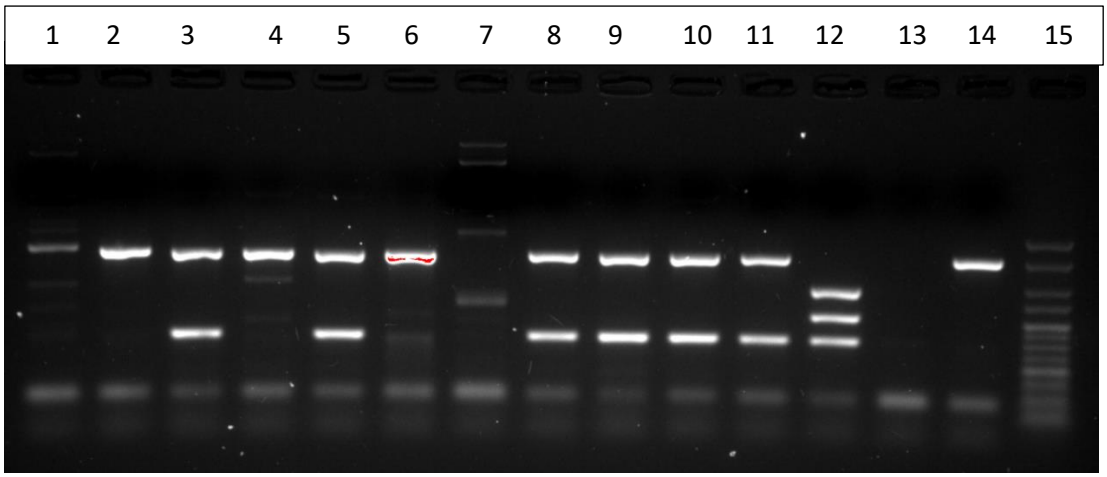
lincoln 2019-03-25_12h31m09s

Lane order and Quadruplex genotype

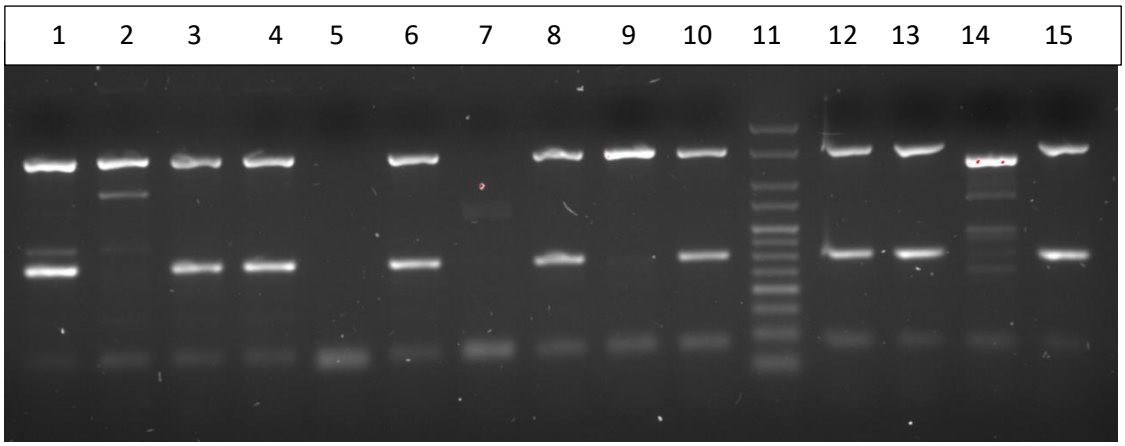
S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	MRD-18-S18	+	-	-	+	B1
2	MRD-13-S18	+	-	-	+	B1
3	MRD-21-S18	+	-	-	+	B1
4	MRD-8-W18	+	-	-	-	A
5	CW-26-A18	+	-	+	-	A/C
6	CW-24-A18	+	-	-	+	B1
7	CW-30-A18	+	-	-	+	B1
8	CW-17-A18	+	-	-	+	B1
9	TL-25-S18	+	-	-	+	B1
10	CW-45-A18	+	-	-	+	B1
11	MRD-60-A18					Not assignable
12	Hyper ladder v (Marker)					
13	TL-11-W18	+	-	-	+	B1
14	TL-10-W18	+	-	-	+	B1
15	MRD-22-S18	+	-	-	+	B1



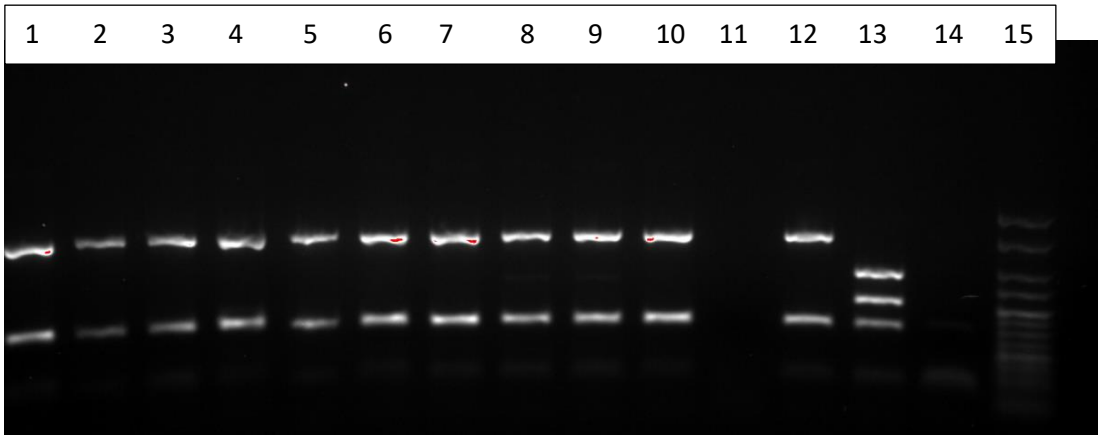
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-23-S18	+	-	-	+	B1
2	MRD-24-S18	+	-	-	+	B1
3	MRD-12-S18	+	-	-	+	B1
4	MRD-11-S18	+	-	-	+	B1
5	MRD-19-S18	+	-	-	+	B1
6	TL-17-S18	+	-	-	+	B1
7	TL-16-S18	+	-	-	+	B1
8	TL-23-S18	+	-	-	+	B1
9	TL-24-S18	+	-	-	+	B1
10	TL-27-S18	+	-	-	+	B1
11	MRD-5-S18	+	-	-	+	B1
12	TL-12-W18	+	-	-	+	B1
13	PTC (Positive template control) ATCC	-	+	+	+	B2
14	NTC (Non-template control)					
15	Hyper ladder v (Marker)					



S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	TL-23-W18	+	-	-	-	A
2	TL-3-W18	+	-	-	-	A
3	TL-8-W18	+	-	-	+	B1
4	TL-20-W18	+	-	-	-	A
5	TL-12-S18	+	-	-	+	B1
6	TL-7-W18	+	-	-	-	A
7	TL-8*-W18	-	-	-	-	Unknown
8	TL-27-W18	+	-	-	+	B1
9	TL-14-W18	+	-	-	+	B1
10	TL-16-W18	+	-	-	+	B1
11	TL-24-W18	+	-	-	+	B1
12	PTC (Positive template control) ATCC	-	+	+	+	B2
13	NTC (Non-template control)					
14	TL-1-W18	+	-	-	-	A
15	Hyper ladder v (Marker)					



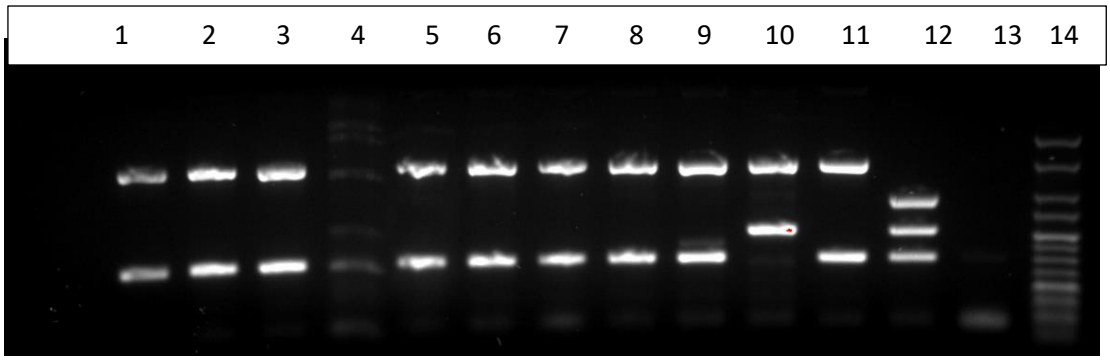
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	CW-19-A18	+	-	-	+	B1
2	TL-28-S18	+	-	-	-	A
3	MRD-1-S18	+	-	-	+	B1
4	TL-26-S18	+	-	-	+	B1
5	TL-25-W18	-	-	-	-	Unknown
6	TL-5-W18	+	-	-	+	B1
7	MRD-8-S18	-	-	-	-	Unknown
8	MRD-17-S18	+	-	-	+	B1
9	TL-2-W18	+	-	-	-	A
10	TL-18-W18	+	-	-	+	B1
11	Hyper ladder v (Marker)					
12	MRD-31-S17	+	-	-	+	B1
13	MRD-21-S17	+	-	-	+	B1
14	TL-50-W18	-	-	+	-	Clade IorII
15	TL-49-W18	+	-	-	+	B1



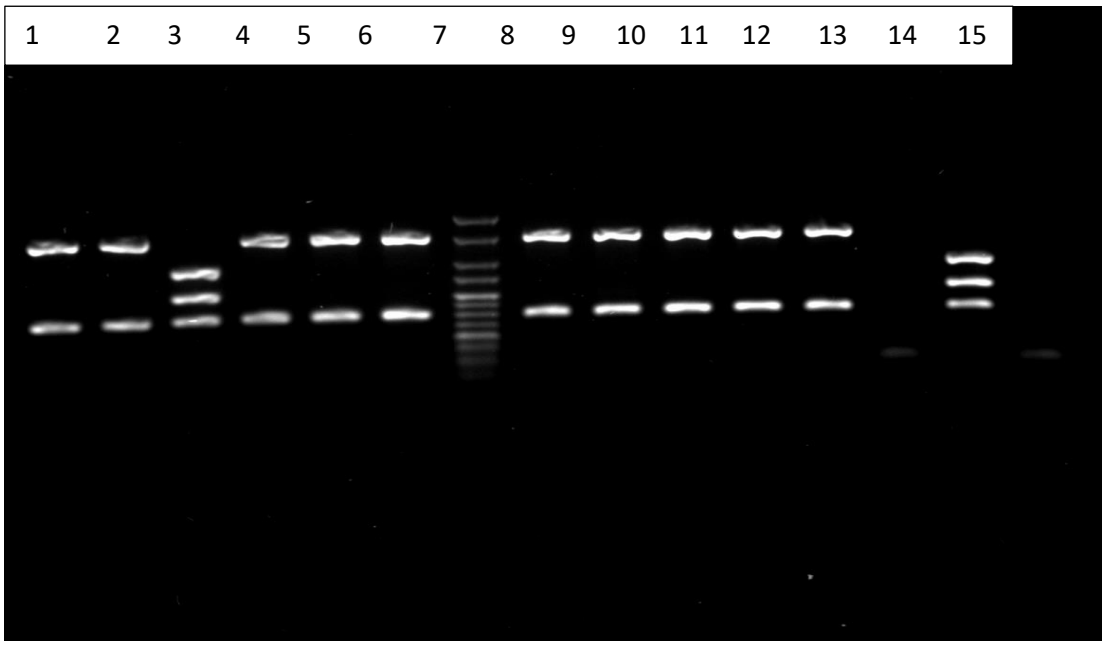
lincoln 2019-03-29_11h40m37s

Lane order and Quadruplex genotype

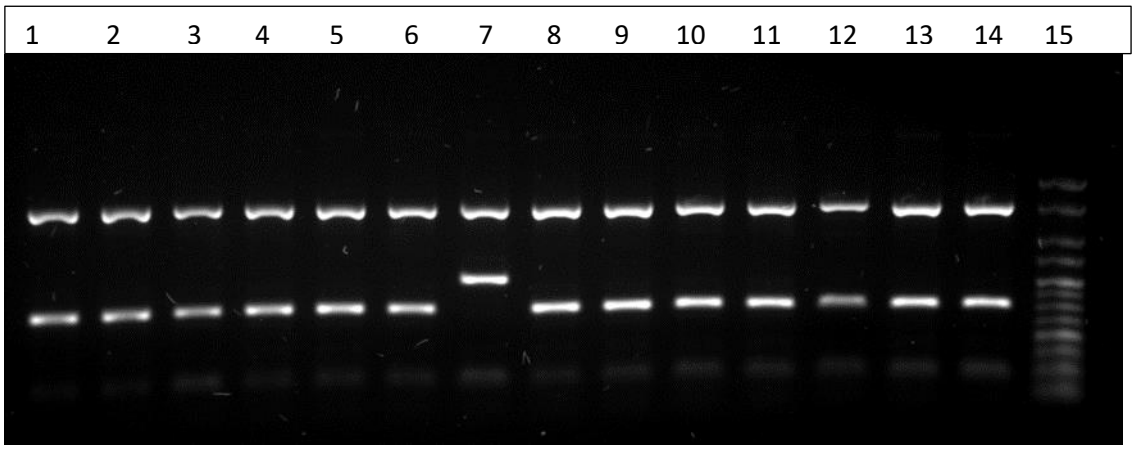
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-22-S17	+	-	-	+	B1
2	MRD-12-S17	+	-	-	+	B1
3	MRD-7-S17	+	-	-	+	B1
4	MRD-5-S17	+	-	-	+	B1
5	MRD-62-S17	+	-	-	+	B1
6	MRD-59-S17	+	-	-	+	B1
7	MRD-30-S17	+	-	-	+	B1
8	MRD-9-S18	+	-	-	+	B1
9	MRD-10-S18	+	-	-	+	B1
10	TL-19-W18	+	-	-	+	B1
11						
12	TL-21-W18	+	-	-	+	B1
13	PTC (Positive template control) ATCC	-	+	+	+	B2
14	NTC (Non-template control)					
15	Hyper ladder v (Marker)					



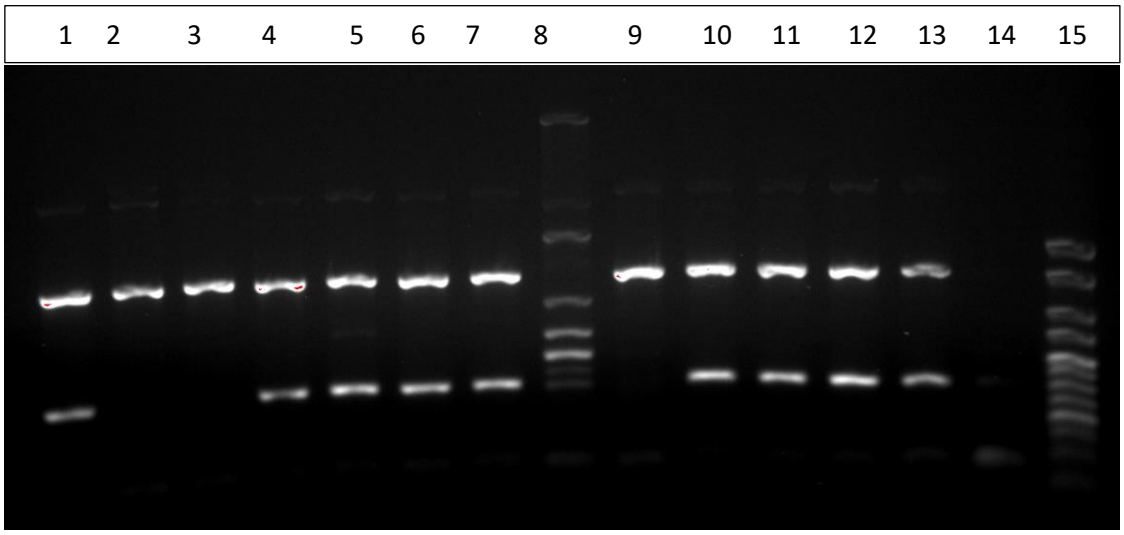
		Quadruplex genotype				Phylogroup
Lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-47-S17	+	-	-	+	B1
2	MRD-61-S17	+	-	-	+	B1
3	TL-32-W18	+	-	-	+	B1
4	TL-39*-W18	+	-	-	+	B1
5	TL-40-W18	+	-	-	+	B1
6	TL-38-W18	+	-	-	+	B1
7	TL-42-W18	+	-	-	+	B1
8	MRD-51-S17	+	-	-	+	B1
9	MRD-60-S17	+	-	-	+	B1
10	MRD-45-S17	+	-	+	-	A/C
11	TL-43-W18	+	-	-	+	B1
12	PTC (Positive template control) ATCC	-	+	+	+	B2
13	NTC (Non-template control)					
14	Hyper ladder v (Marker)					



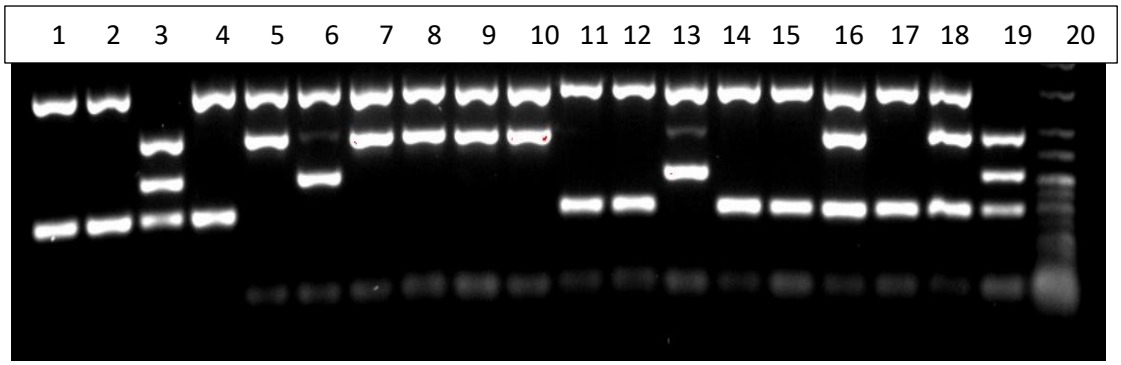
S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	*chu A*	*yja A*	*TspE4C2*	
1	TL-44-W18	+	-	-	+	B1
2	TL-41-W18	+	-	-	+	B1
3	MRD-23-S17	-	+	+	+	B2
4	MRD-58-S17	+	-	-	+	B1
5	MRD-25-S17	+	-	-	+	B1
6	MRD-10-S17	+	-	-	+	B1
7	Hyper ladder v (Marker)					
8	PF-17-W18	+	-	-	+	B1
9	PF-15-W18	+	-	-	+	B1
10	PF-14-W18	+	-	-	+	B1
11	PF-4-W18	+	-	-	+	B1
12	PF-1-W18	+	-	-	+	B1
13	NTC (Non-template control)					
14	PTC (Positive template control) ATCC	-	+	+	+	B2
15	NTC (Non-template control)					



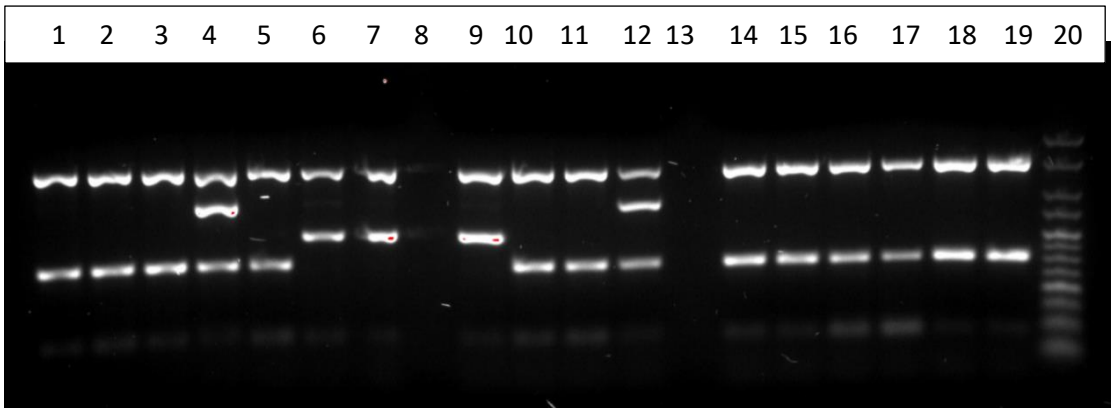
S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	*chu A*	*yja A*	*TspE4C2*	
1	PF-5-W18	+	-	-	+	B1
2	PF-8-W18	+	-	-	+	B1
3	PF-7-W18	+	-	-	+	B1
4	PF-16-W18	+	-	-	+	B1
5	PF-6-W18	+	-	-	+	B1
6	PF-11-W18	+	-	-	+	B1
7	PF-13-W18	+	-	+	-	A/C
8	PF-10-W18	+	-	-	+	B1
9	PF-9-W18	+	-	-	+	B1
10	PF-3-W18	+	-	-	+	B1
11	PF-2-W18	+	-	-	+	B1
12	PF-50-W18	+	-	-	+	B1
13	PF-51-W18	+	-	-	+	B1
14	PF-43-W18	+	-	-	+	B1
15	Hyper ladder v (Marker)					



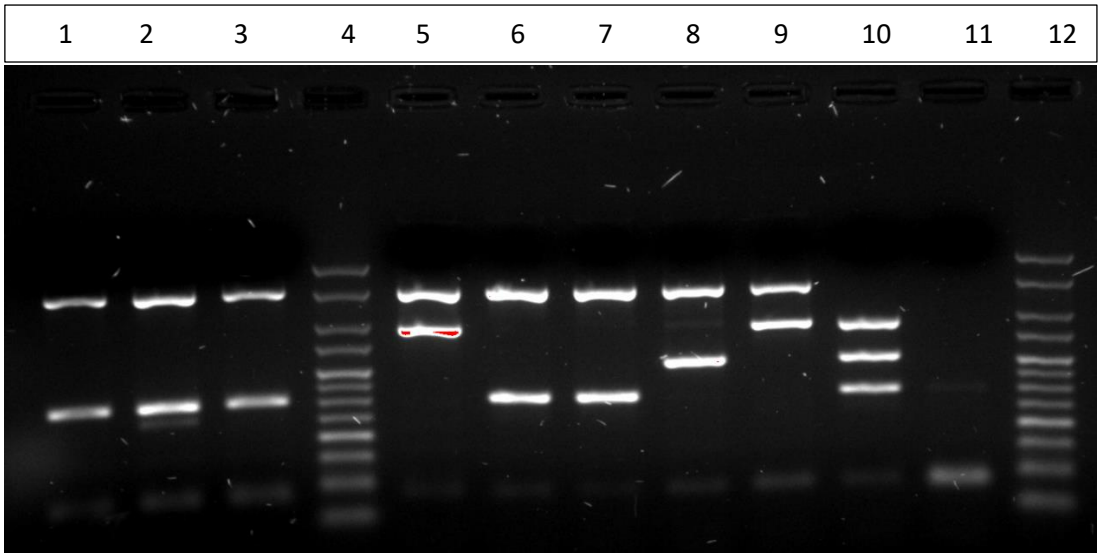
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-34-W18	+	-	-	+	B1
2	PF-53-W18	+	-	-	-	A
3	PF-56-W18	+	-	-	-	A
4	PF-49-W18	+	-	-	+	B1
5	PF-48-W18	+	-	-	+	B1
6	PF-35-W18	+	-	-	+	B1
7	PF-46-W18	+	-	-	+	B1
8	PF-47-W18					Unknown
9	PF-38-W18	+	-	-	-	A
10	PF-36-W18	+	-	-	+	B1
11	PF-33-W18	+	-	-	+	B1
12	CW-4-S18	+	-	-	+	B1
13	CW-1-S18	+	-	-	+	B1
14	NTC					
15	Hyper ladder v (Marker)					



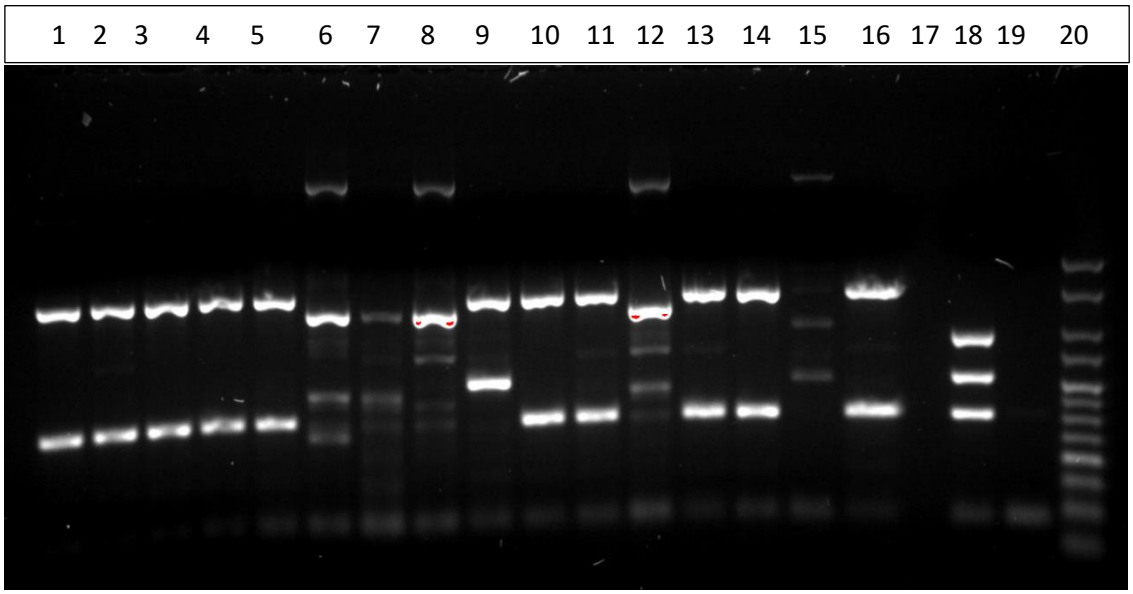
S.No		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	CW-2-S18	+	-	-	+	B1
2	CW-3-S18	+	-	-	+	B1
3	PTC (Positive template control) ATCC	-	+	+	+	B2
4	CW-24-S18	+	-	-	+	B1
5	CW-25-S18	+	+	-	-	D/E
6	CW-20-S18	+	+	+	-	E or clade1
7	CW-30-S18	+	+	-	-	D/E
8	CW-31-S18	+	+	-	-	D/E
9	CW-32-S18	+	+	-	-	D/E
10	CW-8-S18	+	+	-	-	D/E
11	CW-6-S18	+	-	-	+	B1
12	CW-5-S18	+	-	-	+	B1
13	CW-27-S18	+	+	+	-	E or clade1
14	CW-51-S18	+	-	-	+	B1
15	CW-17-S18	+	-	-	+	B1
16	CW-34-S18	+	+	+	-	E or clade1
17	CW-29-S18	+	-	-	+	B1
18	CW-18-S18	+	+	+	-	E or clade1
19	PTC (Positive template control) ATCC	-	+	+	+	B2
20	Hyper ladder v (Marker)					



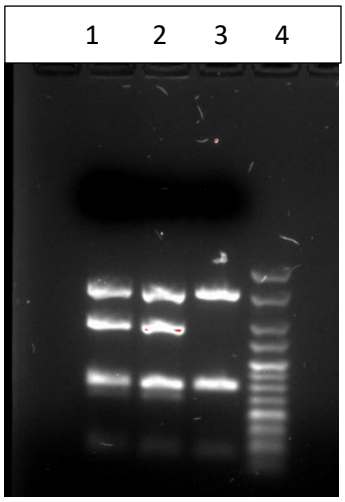
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	CW-11-S18	+	-	-	+	B1
2	CW-12-S18	+	-	-	+	B1
3	CW-9-S18	+	-	-	+	B1
4	CW-35-S18	+	+	-	+	D/E
5	CW-13-S18	+	-	-	+	B1
6	CW-15-S18	+	-	+	-	A/C
7	CW-14-S18	+	-	+	-	A/C
8						
9	CW-7-S18	+	-	+	-	A/C
10	CW-10-S18	+	-	-	+	B1
11	CW-40-S18	+	-	-	+	B1
12	CW-44-S18	+	+	-	+	D/E
13						
14	CW-36-S18	+	-	-	+	B1
15	CW-38-S18	+	-	-	+	B1
16	CW-43-S18	+	-	-	+	B1
17	CW-46-S18	+	-	-	+	B1
18	CW-45-S18	+	-	-	+	B1
19	CW-29-S18	+	-	-	+	B1
20	Hyper ladder v (Marker)					



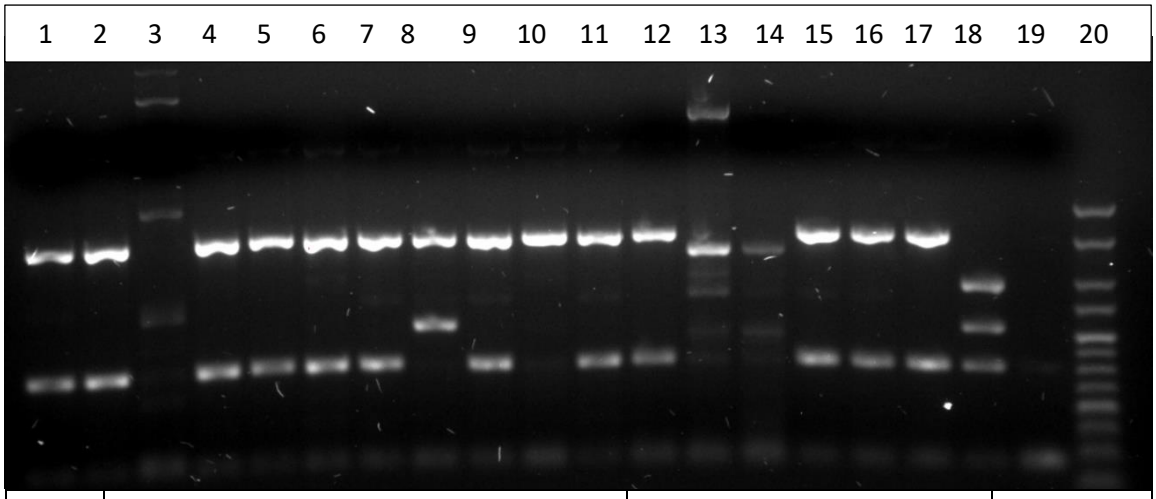
S.No		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	CW-22-S18	+	-	-	+	B1
2	CW-28-S18	+	-	-	+	B1
3	CW-23-S18	+	-	-	+	B1
4	Hyper ladder v (Marker)					
5	CW-39-S18	+	+	-	-	D/E
6	CW-42-S18	+	-	-	+	B1
7	CW-41-S18	+	-	-	+	B1
8	CW-16-S18	+	-	+	-	A/C
9	CW-26-S18	+	+	-	-	D/E
10	PTC (Positive template control) ATCC	-	+	+	+	B2
11	NTC					
12	Hyper ladder v (Marker)					



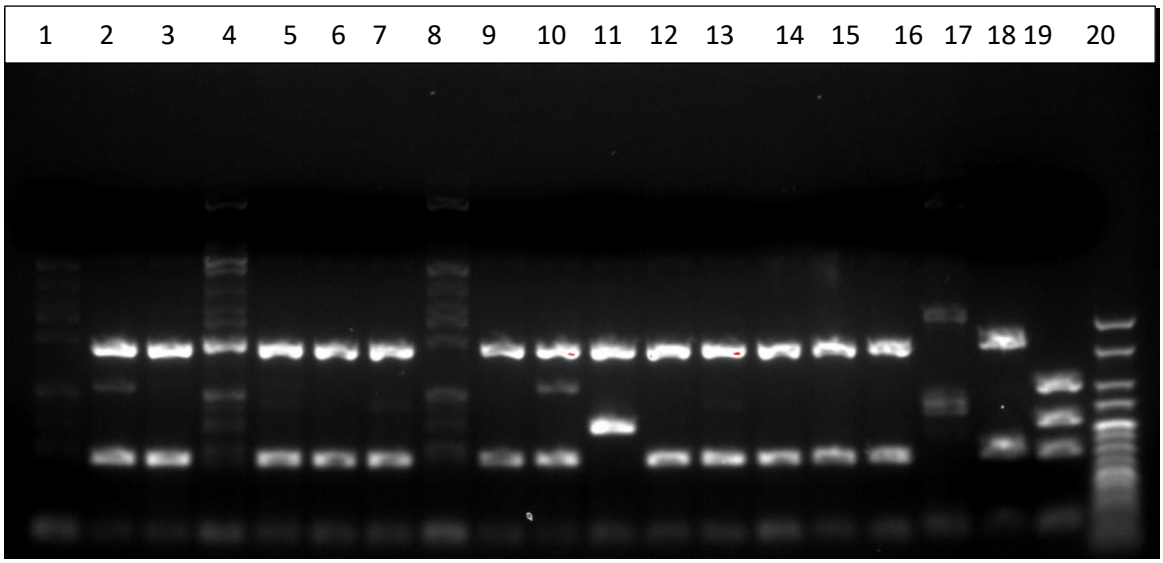
S.No		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	TL-39-A18	+	-	-	+	B1
2	TL-12-A18	+	-	-	+	B1
3	TL-31-A18	+	-	-	+	B1
4	TL-27-A18	+	-	-	+	B1
5	TL-7-A18	+	-	-	+	B1
6	TL-9-A18					Unknown
7	TL-36-A18					Unknown
8	TL-40-A18					Unknown
9	TL-18-A18	+	-	+	-	A/C
10	TL-38-A18	+	-	-	+	B1
11	TL-20-A18	+	-	-	+	B1
12	TL-34-A18					Unknown
13	TL-42-A18	+	-	-	+	B1
14	TL-32-A18	+	-	-	+	B1
15	TL-26-A18	-	-	+	-	Clade I+II
16	TL-43-A18	+	-	-	+	B1
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	NTC					
20	Hyper ladder v (Marker)					



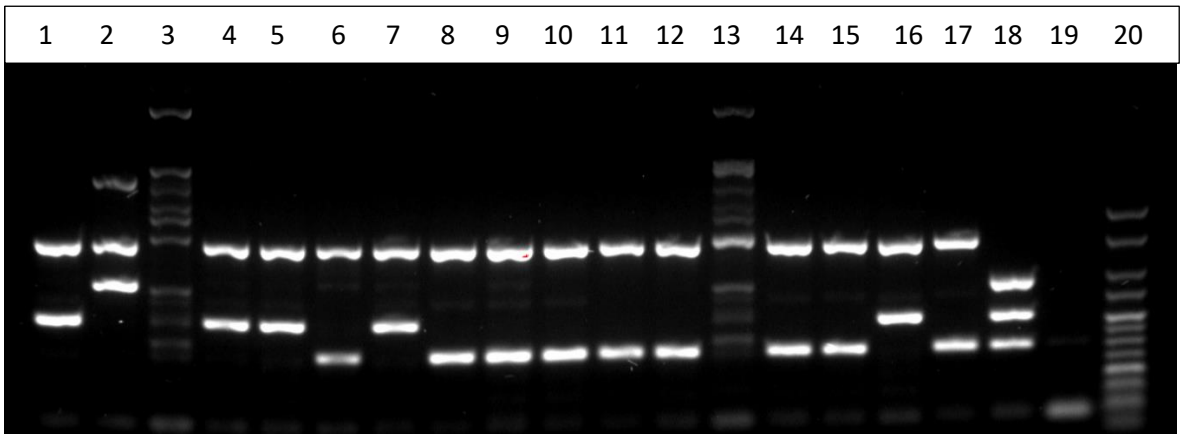
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	CW-34-W18	+	+	-	+	D/E
2	CW-35-W18	+	+	-	+	D/E
3	CW-37-W18	+	-	-	+	B1
20	Hyper ladder v (Marker)					



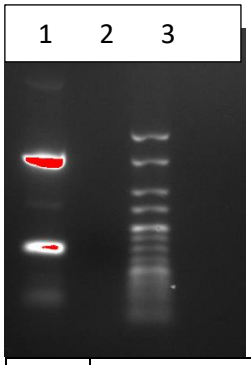
S.No		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	TL-33-A18	+	-	-	+	B1
2	TL-19-A18	+	-	-	+	B1
3	CW-51*-S18					Unknown
4	CW-47-S18	+	-	-	+	B1
5	CW-49-S18	+	-	-	+	B1
6	CW-50-S18	+	-	-	+	B1
7	TL-25-A18	+	-	-	+	B1
8	TL-1-A18	+	-	+	-	A/C
9	TL-11-A18	+	-	-	+	B1
10	TL-24-A18	+	-	-	-	A
11	TL-16-A18	+	-	-	+	B1
12	TL-14-A18	+	-	-	+	B1
13	TL-12*-A18					Unknown
14	TL-48-A18					Unknown
15	TL-52-A18	+	-	-	+	B1
16	TL-50-A18	+	-	-	+	B1
17	TL-13-A18	+	-	-	+	B1
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	NTC					
20	Hyper ladder v (Marker)					



		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	CW-6-W18					Unknown
2	CW-29-W18	+	+	-	+	D/E
3	CW-25-W18	+	-	-	+	B1
4	CW-19-W18	+	-	-	-	A
5	CW-7-W18	+	-	-	+	B1
6	CW-100-S17	+	-	-	+	B1
7	TL-19-A18	+	-	-	+	B1
8	CW-20-W18					Unknown
9	CW-21-W18	+	-	-	+	B1
10	CW-28-W18	+	+	-	+	D/E
11	CW-23-W18	+	-	+	-	A/C
12	CW-22-W18	+	-	-	+	B1
13	CW-30-W18	+	-	-	+	B1
14	CW-31-W18	+	-	-	+	B1
15	CW-32-W18	+	-	-	+	B1
16	CW-24-W18	+	-	-	+	B1
17	CW-9-W18					Unknown
18	CW-8-W18	+	-	-	+	B1
19	PTC (Positive template control) ATCC	-	+	+	+	B2
20	Hyper ladder v (Marker)					

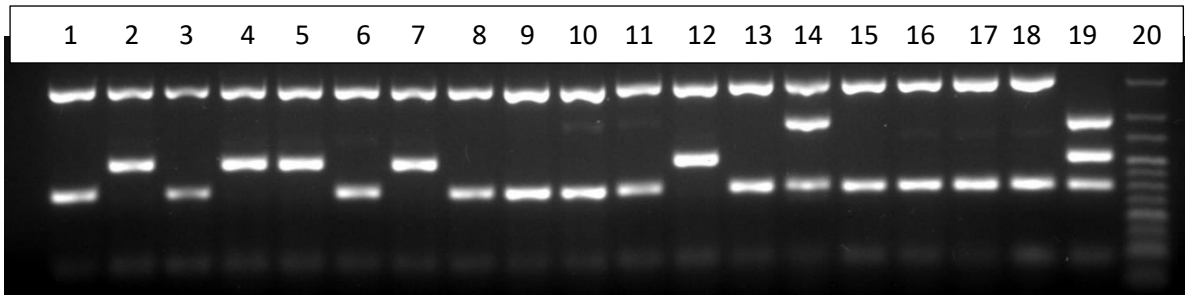


		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-6-S17	+	-	+	-	A/C
2	CW-27-W18	+	+	-	-	D/E
3	CW-14-W18					Unknown
4	PF-9-S17	+	-	+	-	A/C
5	PF-8-S17	+	-	+	-	A/C
6	TL-11-A18	+	-	-	+	B1
7	PF-5-S17	+	-	+	-	A/C
8	CW-5-W18	+	-	-	+	B1
9	CW-4-W18	+	-	-	+	B1
10	CW-3-W18	+	-	-	+	B1
11	TL-33-A18	+	-	-	+	B1
12	TL-17-A18	+	-	-	+	B1
13	CW-2-W18	+	-	-	-	A
14	CW-12-W18	+	-	-	+	B1
15	CW-13-W18	+	-	-	+	B1
16	CW-26-W18	+	-	+	-	A/C
17	CW-17-W18	+	-	-	+	B1
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	NTC					
20	Hyper ladder v (Marker)					

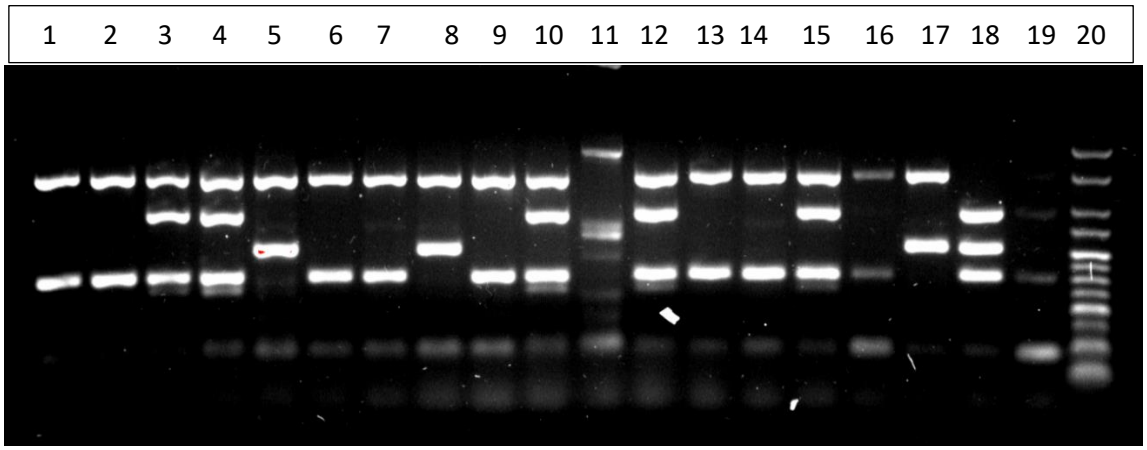


lincoln 2019-04-16_16h52m40s
Lane order and Quadruplex genotype

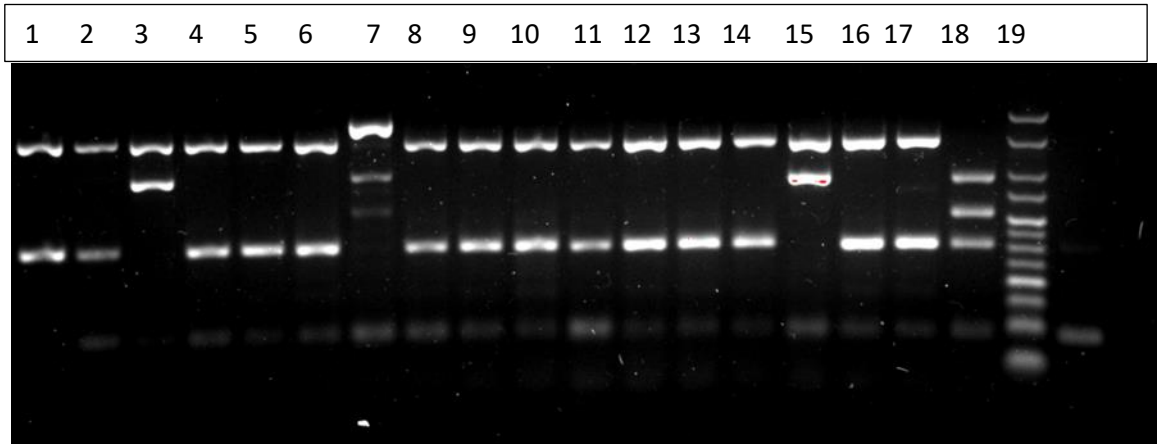
S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	*chu A*	*yja A*	*TspE4C2*	
1	CW-18-W18	+	-	-	+	B1
2	NTC					
3	Hyper ladder v (Marker)					



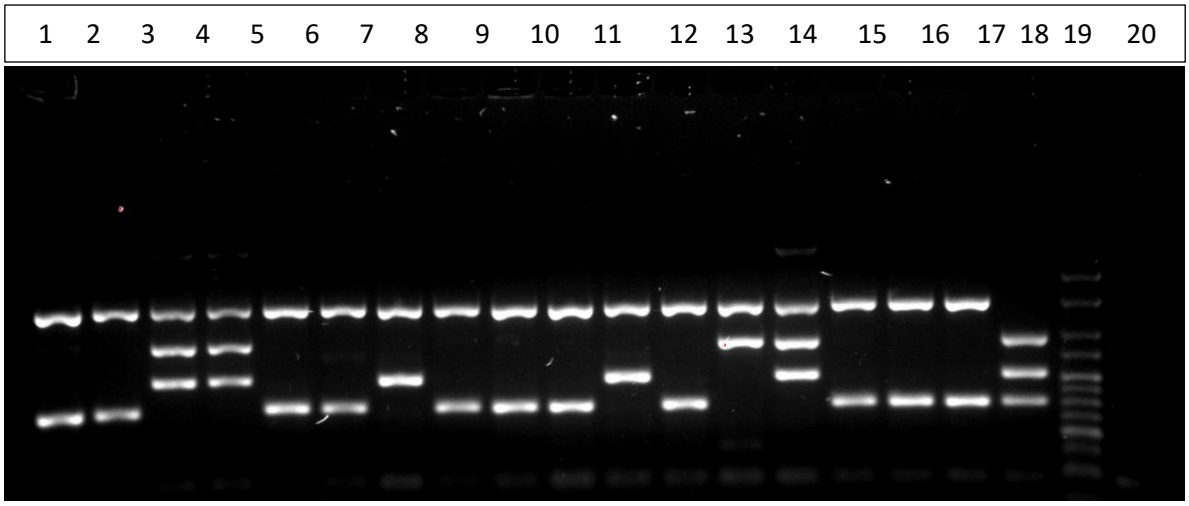
S.No		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-23-S17	+	-	-	+	B1
2	PF-35-S17	+	-	+	-	A/C
3	PF-37-S17	+	-	-	+	B1
4	f	+	-	+	-	A/C
5	PF-34-S17	+	-	+	-	A/C
6	CW-21-W18	+	-	-	+	B1
7	PF-18-S17	+	-	+	-	A/C
8	PF-13-S17	+	-	-	+	B1
9	PF-15-S17	+	-	-	+	B1
10	PF-16-S17	+	-	-	+	B1
11	PF-21-S17	+	-	-	+	B1
12	PF-19-S17	+	-	+	-	A/C
13	PF-22-S17	+	-	-	+	B1
14	CW-49-W18	+	+	-	+	D/E
15	CW-51-W18	+	-	-	+	B1
16	CW-10-W18	+	-	-	+	B1
17	CW-11-W18	+	-	-	+	B1
18	CW-1-W18	+	-	-	+	B1
19	PTC (Positive template control) ATCC	-	+	+	+	B2
20	Hyper ladder v (Marker)					



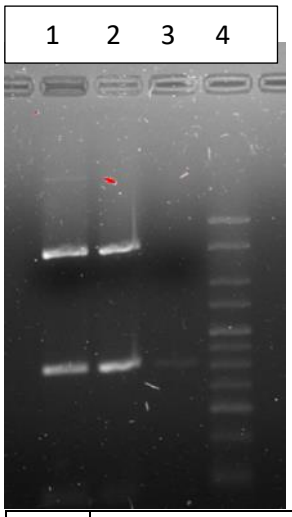
		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	CW-47-W18	+	-	-	+	B1
2	PF-28-S17	+	-	-	+	B1
3	CW-39-W18	+	+	-	+	D/E
4	CW-41-W18	+	+	-	+	D/E
5	PF-30-S17	+	-	+	-	A/C
6	PF-31-S17	+	-	-	+	B1
7	CW-16-W18	+	-	-	+	B1
8	PF-29-S17	+	-	+	-	A/C
9	PF-35-S17	+	-	-	+	B1
10	CW-36-W18	+	+	-	+	D/E
11	CW-15-W18	-	476	-	-	Clade III, IV or V
12	CW-48-W18	+	+	-	+	D/E
13	PF-1-S17	+	-	-	+	B1
14	PF-2-S17	+	-	-	+	B1
15	CW-43-W18	+	+	-	+	D/E
16	CW-40-W18	+	-	-	+	B1
17	PF-27-S17	+	-	+	-	A/C
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Negative template control(NTC)					
20	Hyper ladder v (Marker)					



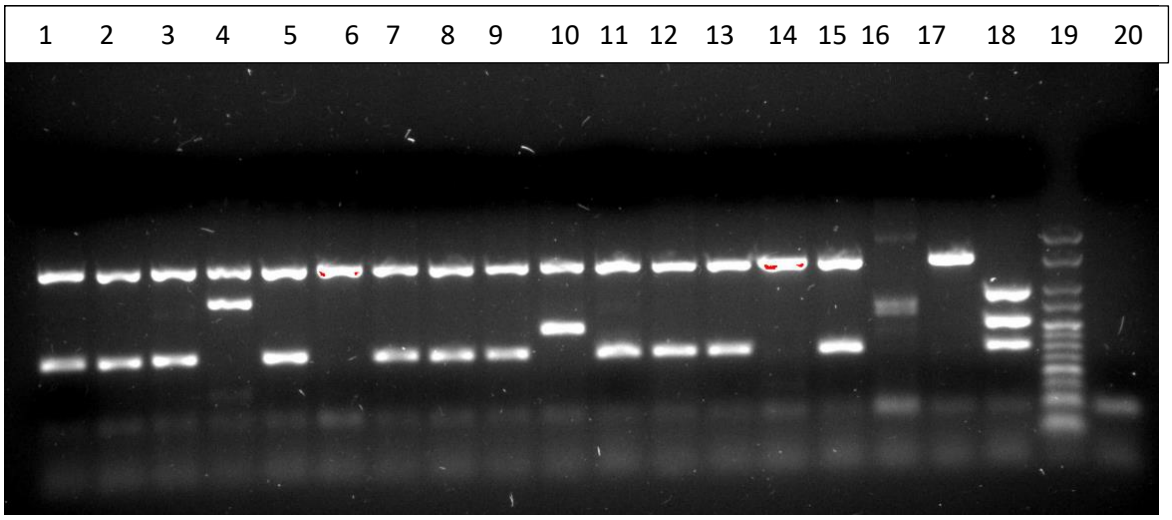
S.No		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	TL-32-S18	+	-	-	+	B1
2	TL-48-S17	+	-	-	+	B1
3	TL-45-S18	+	+	-	-	D/E
4	TL-46-S18	+	-	-	+	B1
5	TL-47-S18	+	-	-	+	B1
6	TL-34-S18	+	-	-	+	B1
7	TL-31-S18	-	476	-	-	Clade III, IV or V
8	TL-33-S18	+	-	-	+	B1
9	TL-30-S18	+	-	-	+	B1
10	TL-29-S18	+	-	-	+	B1
11	TL-42-S18	+	-	-	+	B1
12	TL-52-S18	+	-	-	+	B1
13	TL-49-S18	+	-	-	+	B1
14	TL-50-S18	+	-	-	+	B1
15	TL-51-S18	+	+	-	-	D/E
16	TL-41-S18	+	-	-	+	B1
17	TL-44-S18	+	-	-	+	B1
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					



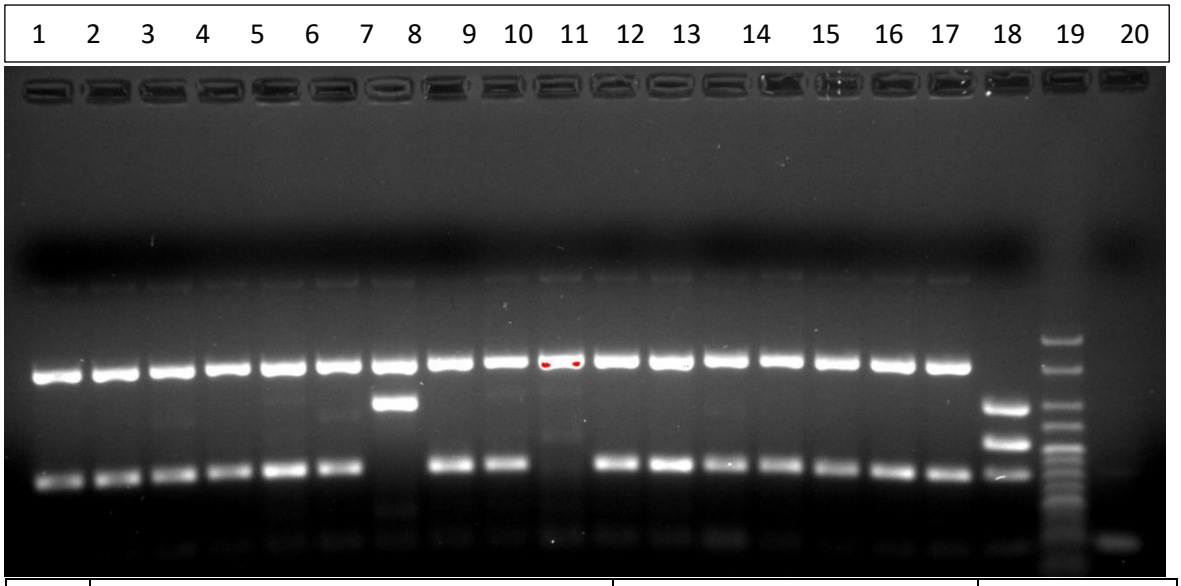
		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	PF-43-S17	+	-	-	+	B1
2	PF-46-S17	+	-	-	+	B1
3	PF-39-S17	+	+	+	-	E or clade I
4	PF-45-S17	+	+	+	-	E or clade I
5	PF-49-S17	+	-	-	+	B1
6	PF-47-S17	+	-	-	+	B1
7	PF-48-S17	+	-	+	-	A/C
8	PF-36-S17	+	-	-	+	B1
9	TL-38-S18	+	-	-	+	B1
10	PF-37-S17	+	-	-	+	B1
11	PF-38-S17	+	-	+	-	A/C
12	PF-35-S17	+	-	-	+	B1
13	MRD-30-S18	+	+	-	-	D/E
14	TL-42-S17	+	+	+	-	E or clade I
15	TL-37-S18	+	-	-	+	B1
16	TL-36-S18	+	-	-	+	B1
17	TL-43-S18	+	-	-	+	B1
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					
20	NTC					



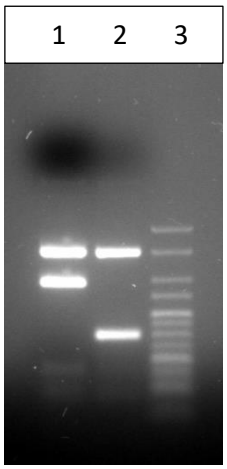
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-25-S17	+	-	-	+	B1
2	CW-45-W18	+	-	-	+	B1
3	CW-42-W18					Unknown
4	Hyper V ladder					



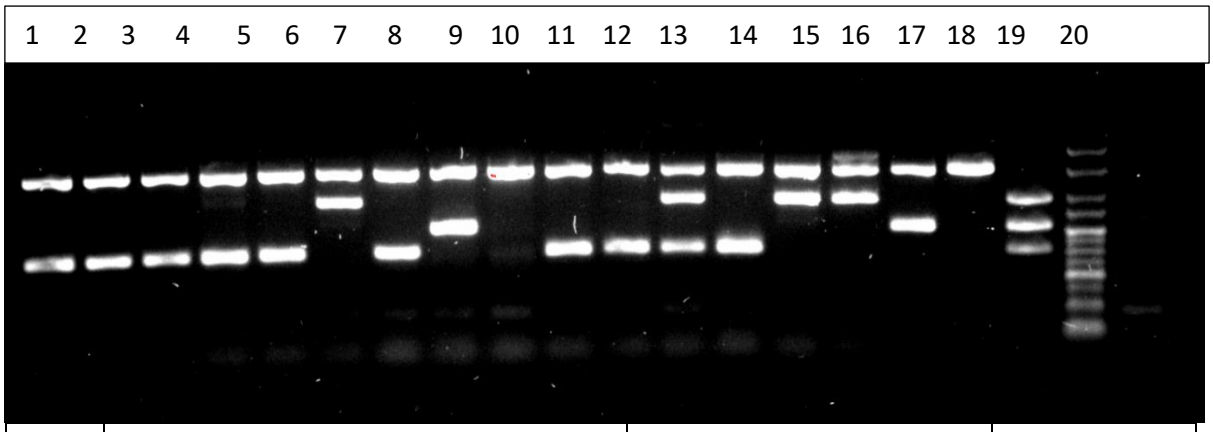
S.No		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	MRD-52-S18	+	-	-	+	B1
2	MRD-34-S18	+	-	-	+	B1
3	MRD-36-S18	+	-	-	+	B1
4	MRD-38-S18	+	+	-	-	D/E
5	MRD-49-S18	+	-	-	+	B1
6	MRD-53-S18	+	-	-	-	A
7	MRD-54-S18	+	-	-	+	B1
8	MRD-55-S18	+	-	-	+	B1
9	MRD-44-S18	+	-	-	+	B1
10	MRD-47-S18	+	-	+	-	A/C
11	MRD-45-S18	+	-	-	+	B1
12	MRD-43-S18	+	-	-	+	B1
13	MRD-39-S18	+	-	-	+	B1
14	MRD-40-S18	+	-	-	-	A
15	MRD-32-S18	+	-	-	+	B1
16	MRD-46-S18					Unknown
17	MRD-29-S18	+	-	-	+	B1
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					
20	NTC					



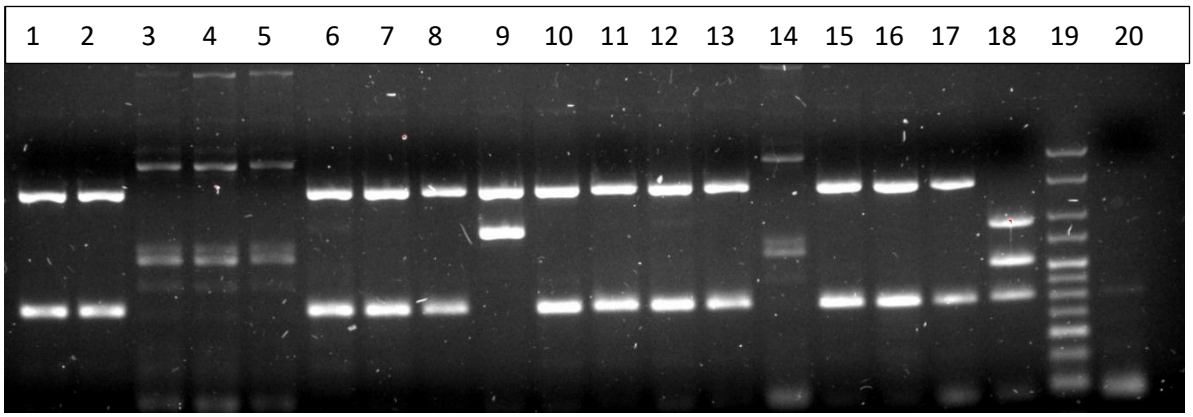
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-42-S17	+	-	-	+	B1
2	MRD-10-S17	+	-	-	+	B1
3	MRD-37-S17	+	-	-	+	B1
4	MRD-29-S17	+	-	-	+	B1
5	MRD-47-S17	+	-	-	+	B1
6	MRD-36-S17	+	-	-	+	B1
7	MRD-41-S18	+	+	-	-	D/E
8	MRD-57-S17	+	-	-	+	B1
9	MRD-65-S17	+	-	-	+	B1
10	MRD-13-S17	+	-	-	-	A
11	MRD-41-S17	+	-	-	+	B1
12	MRD-43-S17	+	-	-	+	B1
13	MRD-30-S17	+	-	-	+	B1
14	MRD-18-S17	+	-	-	+	B1
15	MRD-32-S17	+	-	-	+	B1
16	MRD-26-S17	+	-	-	+	B1
17	MRD-35-S17	+	-	-	+	B1
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					
20	NTC					



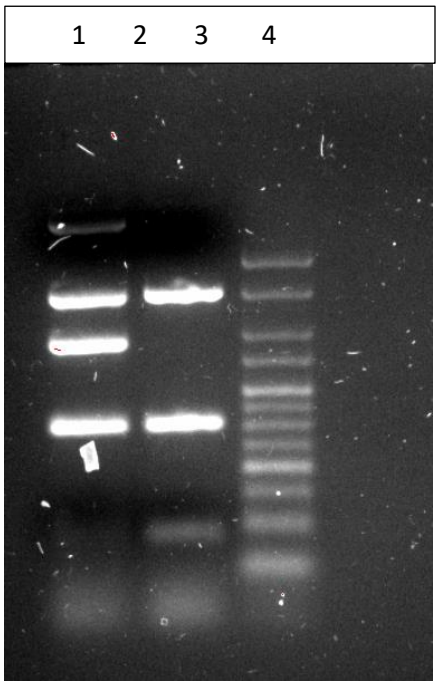
S.No		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	MRD-31-S18	+	+	-	-	D/E
2	MRD-50-S17	+	-	-	+	B1
3	Hyper ladder v (Marker)					



S.No		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MRD-54-S17	+	-	-	+	B1
2	MRD-26-S17	+	-	-	+	B1
3	MRD-5-S17	+	-	-	+	B1
4	MRD-15-S17	+	-	-	+	B1
5	MRD-11-S17	+	-	-	+	B1
6	MRD-8-S17	+	+	-	-	D/E
7	MRD-55-S18	+	-	-	+	B1
8	MRD-27-S17	+	-	+	-	A/C
9	MRD-28-S17	+	-	-	-	A
10	MRD-20-S17	+	-	-	+	B1
11	MRD-56-S17	+	-	-	+	B1
12	MRD-53-S17	+	+	-	+	D/E
13	MRD-7-S17	+	-	-	+	B1
14	MRD-8-S17	+	+	-	-	D/E
15	MRD-40*-S17	+	+	-	-	D/E
16	MRD-44-S17	+	-	+	-	A/C
17	MRD-31-S17	+	-	-	-	A
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					
20	NTC					



		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	TL-9-S17	+	-	-	+	B1
2	TL-3-S17	+	-	-	+	B1
3	TL-27-S17					Unknown
4	TL-29-S17					Unknown
5	TL-31-S17					Unknown
6	TL-46-S17	+	-	-	+	B1
7	TL-49-S17	+	-	-	+	B1
8	TL-35-S17	+	-	-	+	B1
9	TL-34-S17	+	+	-	-	D/E
10	TL-24-S17	+	-	-	+	B1
11	TL-12-S17	+	-	-	+	B1
12	TL-21-S17	+	-	-	+	B1
13	TL-17-S17	+	-	-	+	B1
14	TL-8-S17					Unknown
15	TL-4-S17	+	-	-	+	B1
16	MR-33-S17	+	-	-	+	B1
17	MR-9-S17	+	-	-	+	B1
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					
20	NTC					



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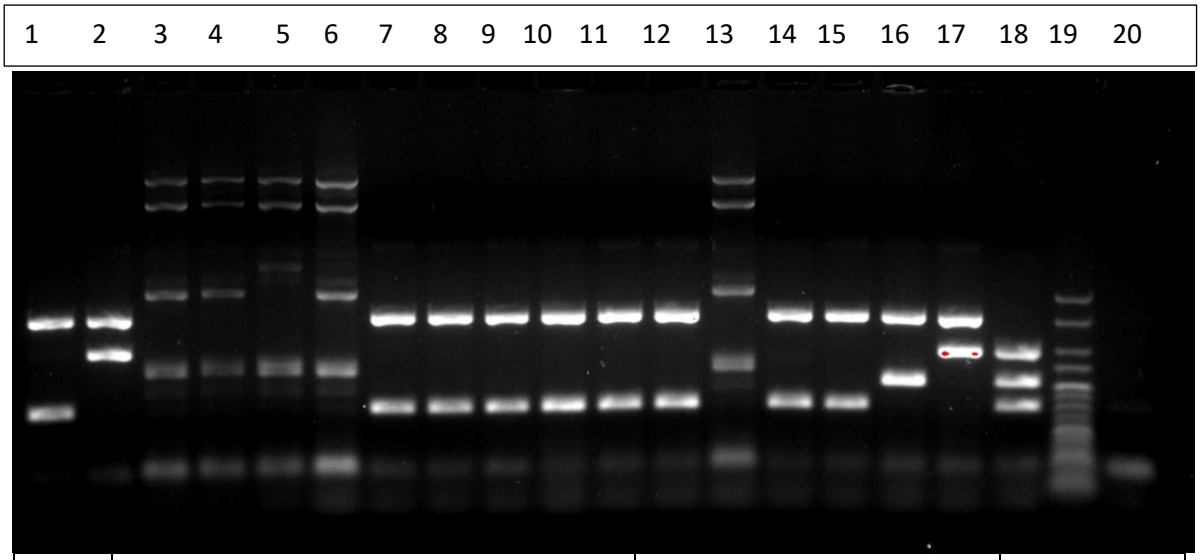
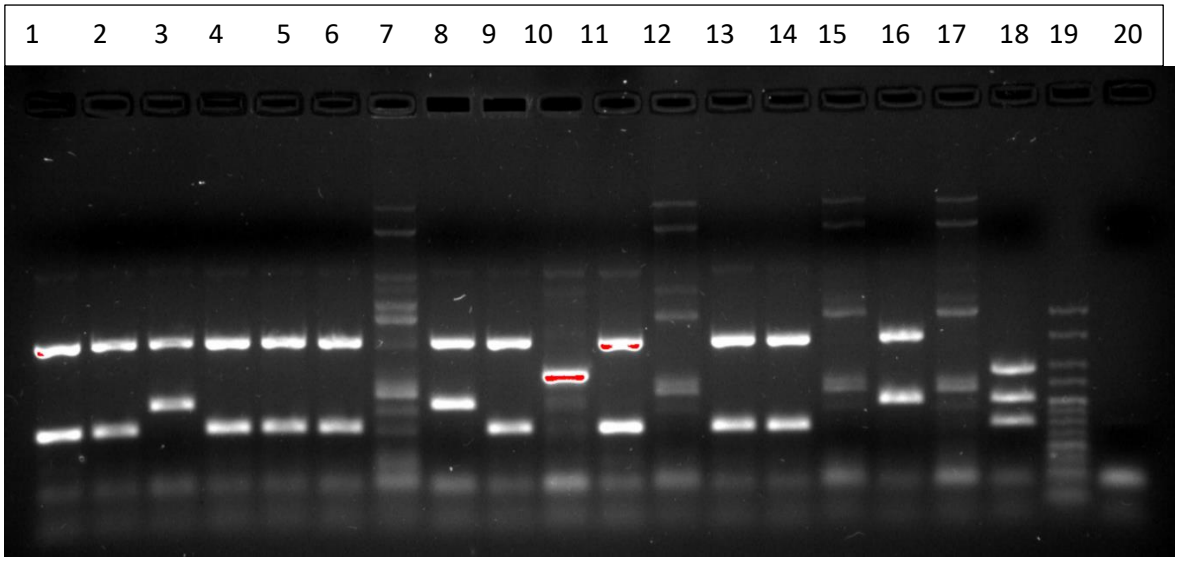
Lane order and Quadruplex genotype

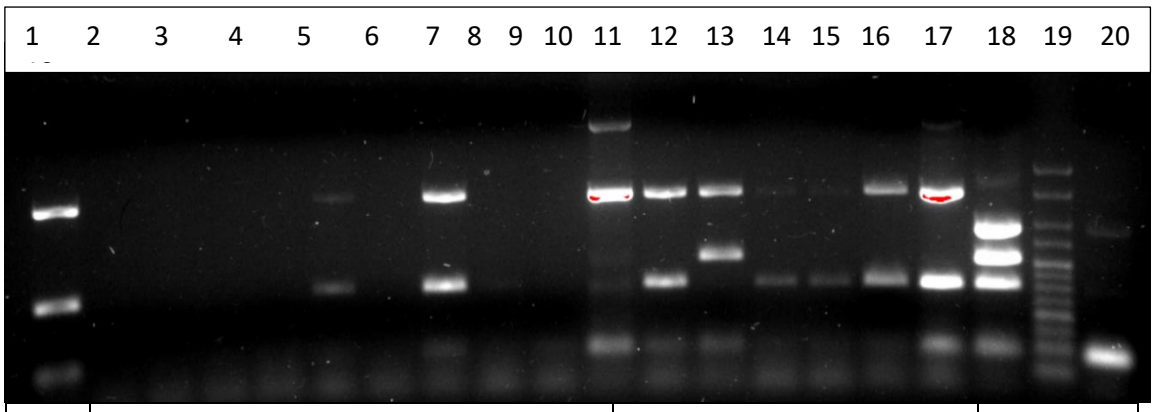
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	MR-51-S17	+	+	-	+	D/E
2	MR-2-S17	+	-	-	+	B1
3	Hyper ladder v (Marker)					



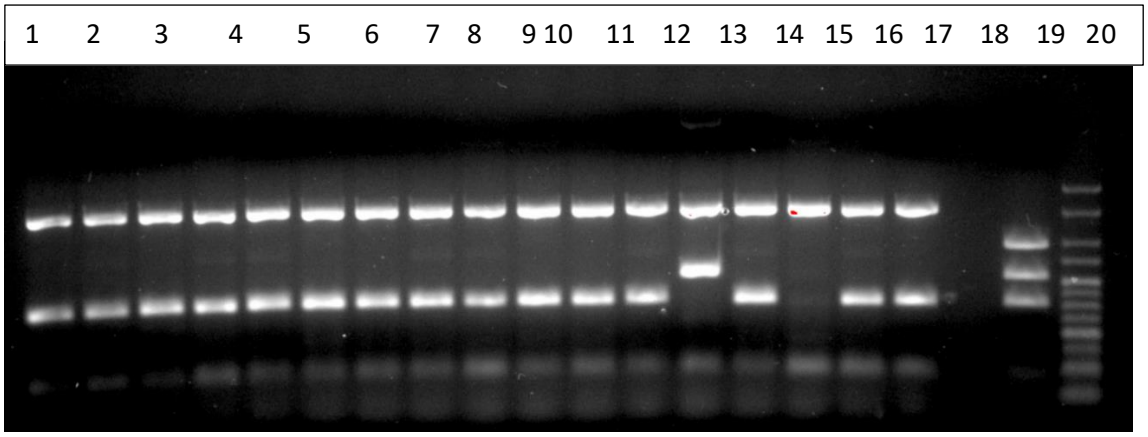
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	TL-7-S17	+	-	-	+	B1
2	TL-32-S17	+	+	-	-	D/E
3	TL-25-S17					Unknown
4	TL-19-S17					Unknown
5	TL-26-S17					Unknown
6	TL-22-S17					Unknown
7	TL-18-S17	+	-	-	+	B1
8	TL-10-S17	+	-	-	+	B1
9	TL-36-S17	+	-	-	+	B1
10	TL-1-S17	+	-	-	+	B1
11	TL-16-S17	+	-	-	+	B1
12	TL-35-S17	+	-	-	+	B1
13	TL-44-S17					Unknown
14	TL-6-S17	+	-	-	+	B1
15	TL-28-S17	+	-	-	+	B1
16	TL-5-S17	+	-	+	-	A/C
17	TL-40-S17	+	+	-	-	D/E
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					
20	NTC					



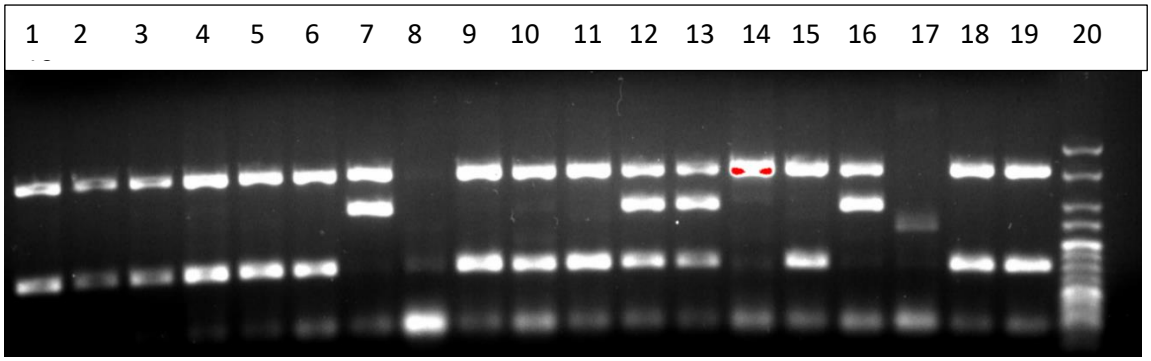
		Quadruplex genotype				Phylogroup
lane	Strain Name	*ArpA*	*chu A*	*yja A*	*TspE4C2*	
1	TL-45-S17	+	-	-	+	B1
2	TL-23-S17	+	-	-	+	B1
3	TL-14-S17	+	-	+	-	A/C
4	TL-38-S17	+	-	-	+	B1
5	TL-46-S17	+	-	-	+	B1
6	TL-20-S17	+	-	-	+	B1
7	TL-50-S17					Unknown
8	TL-11-S17	+	-	+	-	A/C
9	TL-41-S17	+	-	-	+	B1
10	TL-49*-S17	-	+	-	-	F
11	TL-43-S17	+	-	-	+	B1
12	TL-36*-S17					Unknown
13	TL-35-S17	+	-	-	+	B1
14	TL-47-S17	+	-	-	+	B1
15	TL-37-S17					Unknown
16	TL-5-S17	+	-	+	-	A+C
17	TL-42*-S17					Unknown
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					
20	NTC					



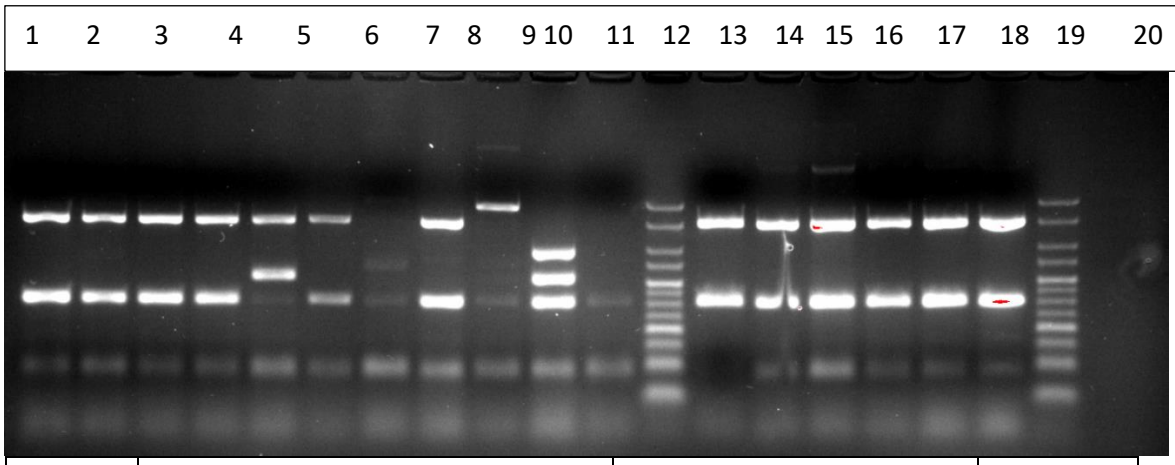
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	TL-8-S18	+	-	-	+	B1
2	TL-9-S18					Unknown
3	TL-6-S18					Unknown
4	TL-7-S18					Unknown
5	TL-18-S18					Unknown
6	TL-17-S18	+	-	-	+	B1
7	TL-12-S18					Unknown
8	TL-11-S18	+	-	-	+	B1
9	TL-10-S18					Unknown
10	TL-16-S17					Unknown
11	TL-48-S17	+	-	-	-	A
12	TL-20-S18	+	-	-	+	B1
13	TL-2-S17	+	-	+	-	A/C
14	TL-12-S17	+	-	-	+	B1
15	TL-24-S17	+	-	-	+	B1
16	TL-41-S17	+	-	-	+	B1
17	TL-51-S17	+	-	-	+	B1
18	PTC (Positive template control) ATCC	-	+	+	+	B2
19	Hyper ladder v (Marker)					
20	NTC					



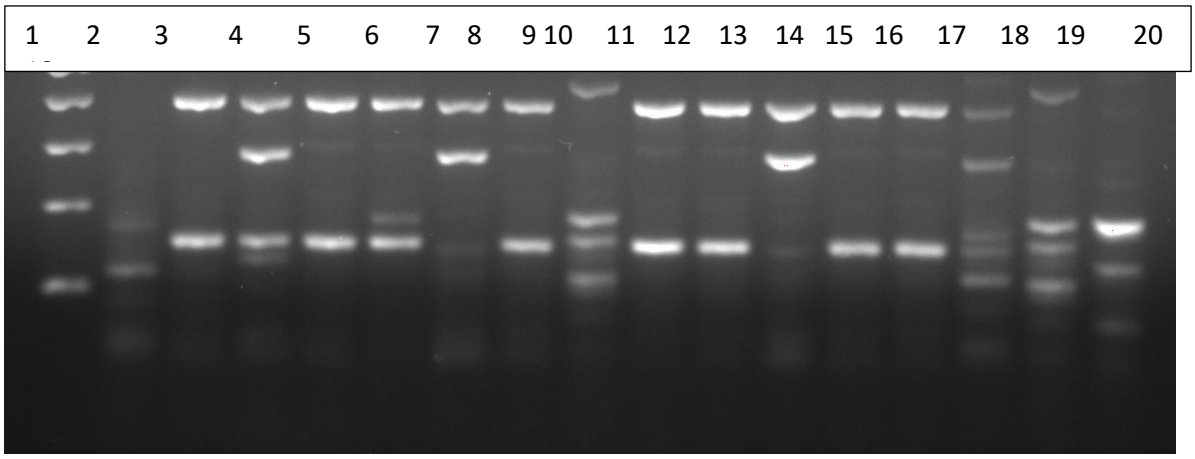
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-1-S18	+	-	-	+	B1
2	PF-4-S18	+	-	-	+	B1
3	PF-15-S18	+	-	-	+	B1
4	PF-2-S18	+	-	-	+	B1
5	PF-3-S18	+	-	-	+	B1
6	PF-7-S18	+	-	-	+	B1
7	PF-10-S18	+	-	-	+	B1
8	PF-14-S18	+	-	-	+	B1
9	PF-13-S18	+	-	-	+	B1
10	PF-16-S18	+	-	-	+	B1
11	PF-11-S18	+	-	-	+	B1
12	PF-12-S18	+	-	-	+	B1
13	PF-9-S18	+	-	+	-	A/C
14	PF-6-S18	+	-	-	+	B1
15	PF-5-S18	+	-	-	-	A
16	PF-8-S18	+	-	-	+	B1
17	TL-4-S18	+	-	-	+	B1
18						
19	PTC (Positive template control) ATCC	-	+	+	+	B2
20	Hyper ladder v (Marker)					



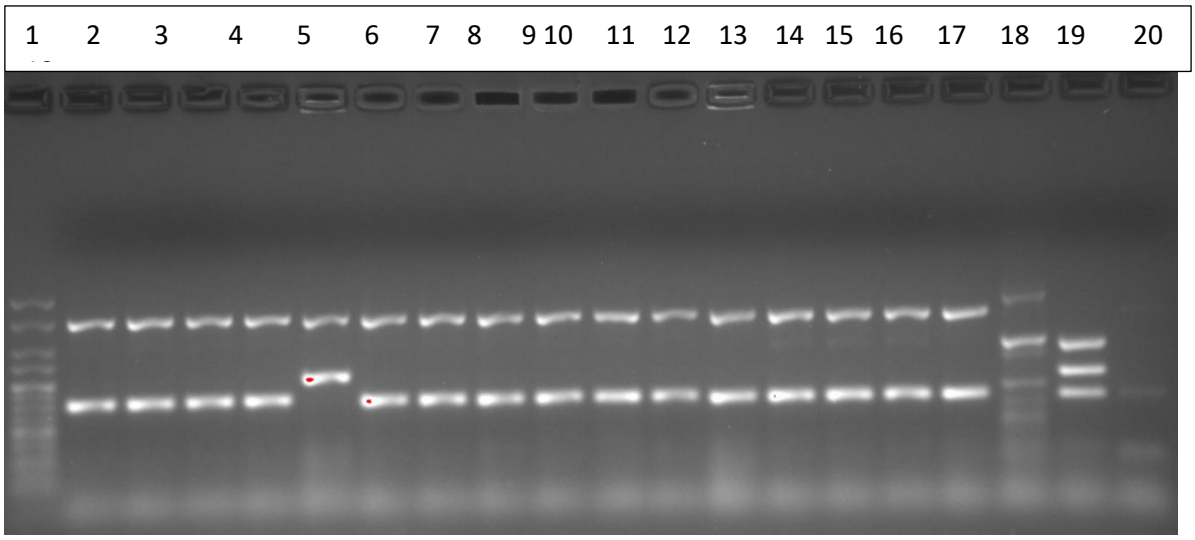
		Quadruplex genotype				Phylogroup
lane	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-17-S18	+	-	-	+	B1
2	PF-24-S18	+	-	-	+	B1
3	PF-23-S18	+	-	-	+	B1
4	PF-22-S18	+	-	-	+	B1
5	PF-21-S18	+	-	-	+	B1
6	PF-18-S18	+	-	-	+	B1
7	PF-19-S18	+	+	-	-	D/E
8	PF-32-S18					Unknown
9	PF-33-S18	+	-	-	+	B1
10	PF-38-S18	+	-	-	+	B1
11	PF-50-S18	+	-	-	+	B1
12	PF-41-S18	+	+	-	+	D/E
13	PF-29-S18	+	+	-	+	D/E
14	PF-27-S18	+	-	-	-	A
15	PF-45-S18	+	-	-	+	B1
16	PF-37-S18	+	+	-	-	D/E
17	PF-31*-S18					Unknown
18	PF-31-S18	+	-	-	+	B1
19	PF-30-S18	+	-	-	+	B1
20	Hyper ladder v (Marker)					



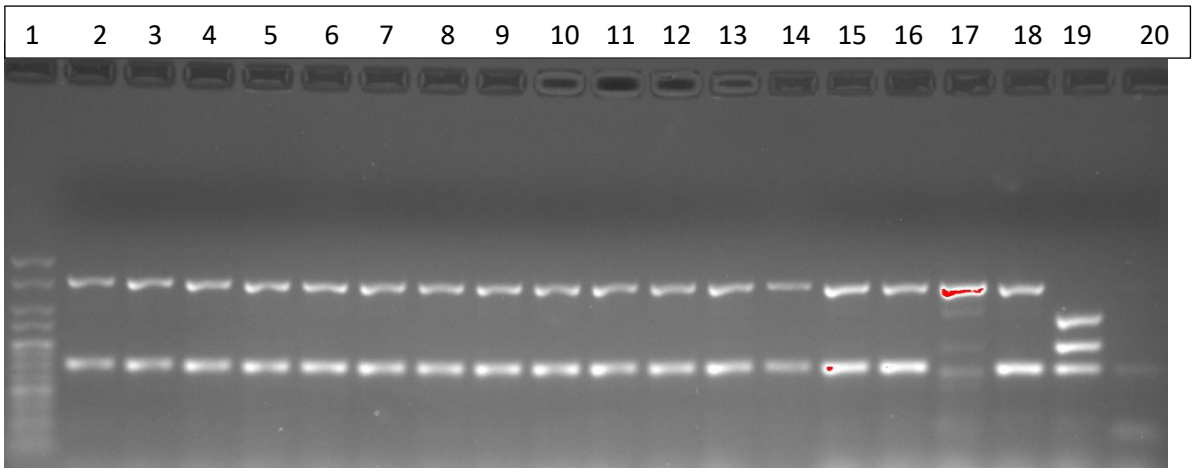
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	PF-43-S18	+	-	-	+	B1
2	PF-47-S18	+	-	-	+	B1
3	PF-28-S18	+	-	-	+	B1
4	PF-53-S18	+	-	-	+	B1
5	PF-56-S18	+	-	+	-	A/C
6	PF-28-S18	+	-	-	+	B1
7	PF-26-S18					Unknown
8	PF-25-S18	+	-	-	+	B1
9	PF-20-S18	-	476	-	-	Clade III, IV or V
10	PTC (Positive template control) ATCC	-	+	+	+	B2
11	NTC					
12	Hyper ladder v (Marker)					
13	TL-21-S17	+	-	-	+	B1
14	TL-51-S17	+	-	-	+	B1
15	TL-16-S17	+	-	-	+	B1
16	TL-5-S18	+	-	-	+	B1
17	TL-3-S18	+	-	-	+	B1
18	TL-1-S18	+	-	-	+	B1
19	Hyper ladder v (Marker)					



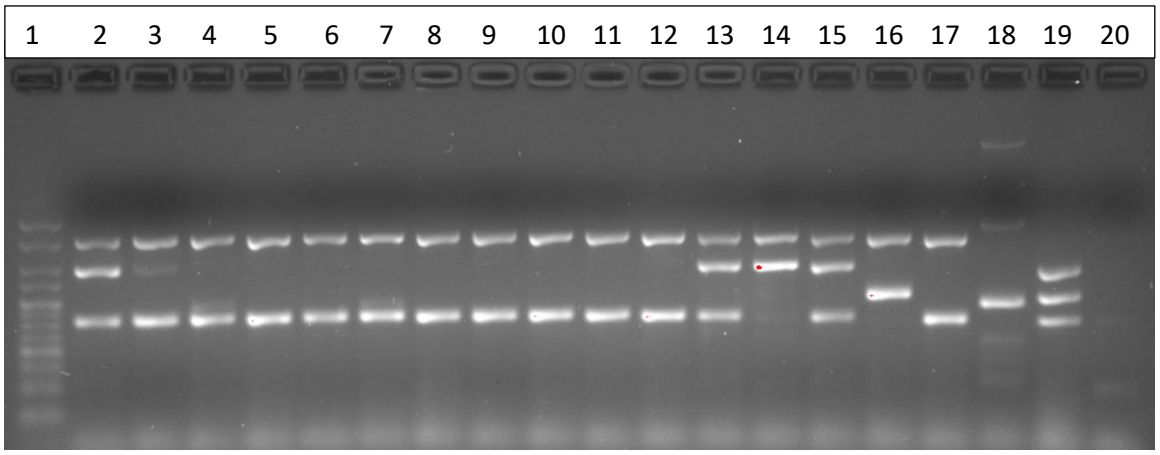
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
Hyper v ladder	Kb+ladder					
2	PF-56*-A18	-	-	-	-	unknown
3	PF-5-A18	+	-	-	+	B1
4	PF-22-A18	+	+	-	+	D/E
5	PF-4-A18	+	-	-	+	B1
6	PF-16-A18	+	-	-	+	B1
7	PF-50-A18	+	+	-	-	D/E
8	PF-60-A18	+	-	-	+	B1
9	PF-10-A18	-	-	+	-	Clad I or II
10	PF-63-A18[b]	+	-	-	+	B1
11	PF-31-A18	+	-	-	+	B1
12	PF-61-A18	+	+	-	-	D/E
13	PF-62-A18	+	-	-	+	B1
14	PF-15-A18	+	-	-	+	B1
15	PF-34-A18	+	-	-	+	B1
16	PF-23-A18	-	-	+	+	unknown
17	Contaminant	-	-	+	-	unknown
18						
19						



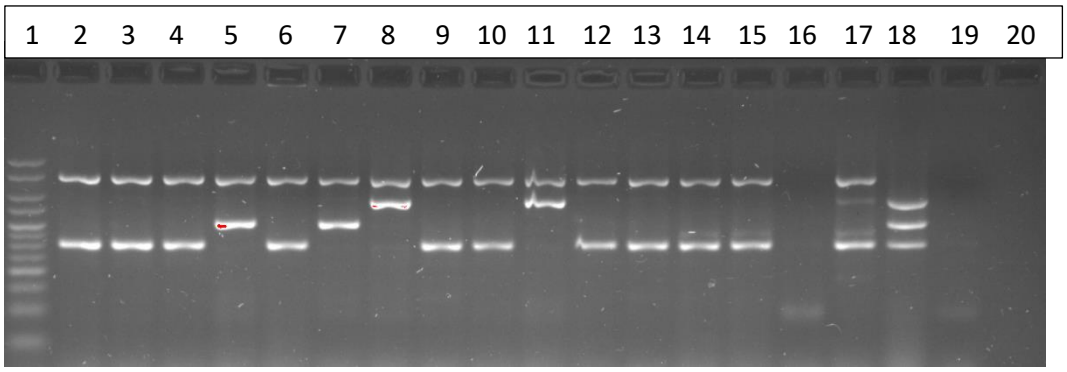
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	TL-22-W18	+	-	-	+	B1
3	TL-19-W18	+	-	-	+	B1
4	TL-21-S18	+	-	-	+	B1
5	TL-14-S18	+	-	-	+	B1
6	TL-39-S17	+	-	+	-	A/C
7	TL-46-S17	+	-	-	+	B1
8	TL-48-S18	+	-	-	+	B1
9	TL-15-W18	+	-	-	+	B1
10	TL-17-W18	+	-	-	+	B1
11	TL-4-W18	+	-	-	+	B1
12	TL-7-W18	+	-	-	+	B1
13	TL-11-S17	+	-	-	+	B1
14	TL-52-S17	+	-	-	+	B1
15	TL-54-S17	+	-	-	+	B1
16	TL-53-S17	+	-	-	+	B1
17	TL-53-S18	+	-	-	+	B1
18	TL-26-W18	-	476	-	-	Clade III, IV or V
19	*E. coli* ATCC25922	-	+	+	+	B2
20	dH_2O					



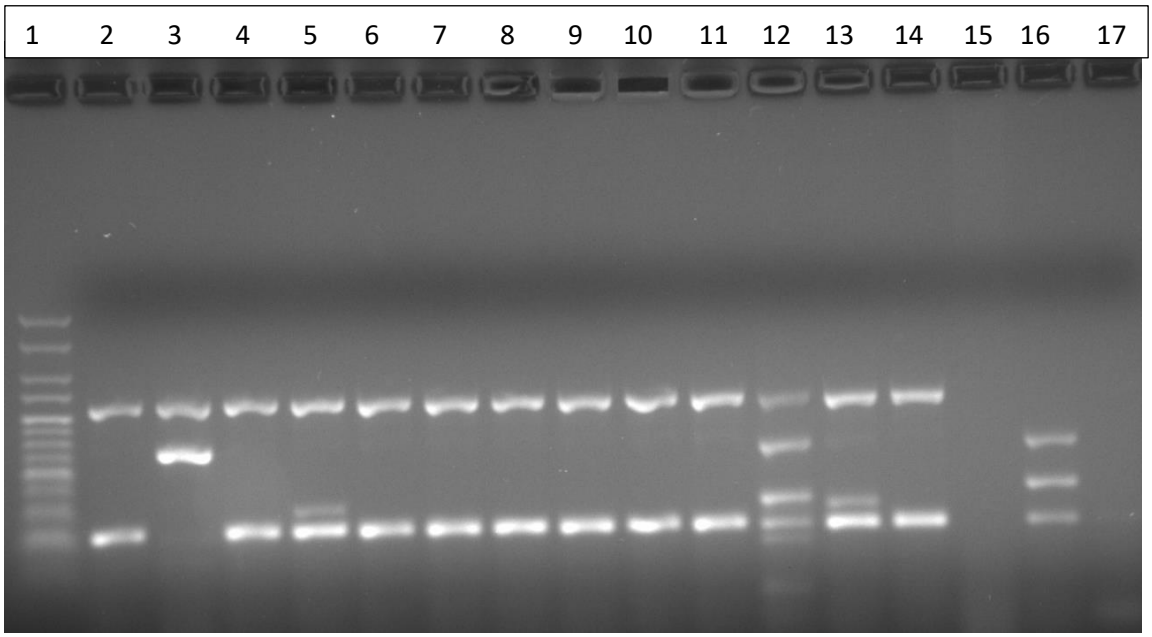
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	TL-45-W18	+	-	-	+	B1
3	TL-48-W18	+	-	-	+	B1
4	TL-47-W18	+	-	-	+	B1
5	TL-46-W18	+	-	-	+	B1
6	TL-9-W18	+	-	-	+	B1
7	TL-19-S18	+	-	-	+	B1
8	TL-27-S17	+	-	-	+	B1
9	TL-30-S17	+	-	-	+	B1
10	TL-33-W18	+	-	-	+	B1
11	TL-31-W18	+	-	-	+	B1
12	TL-30-W18	+	-	-	+	B1
13	TL-29-W18	+	-	-	+	B1
14	TL-35-W18	+	-	-	+	B1
15	TL-38-S18	+	-	-	+	B1
16	TL-55-S18	+	-	-	+	B1
17	TL-13-W18	+	-	-	-	A
18	TL-54-S18	+	-	-	+	B1
19	*E. coli* ATCC25922	-	+	+	+	B2
20	dH_2O					



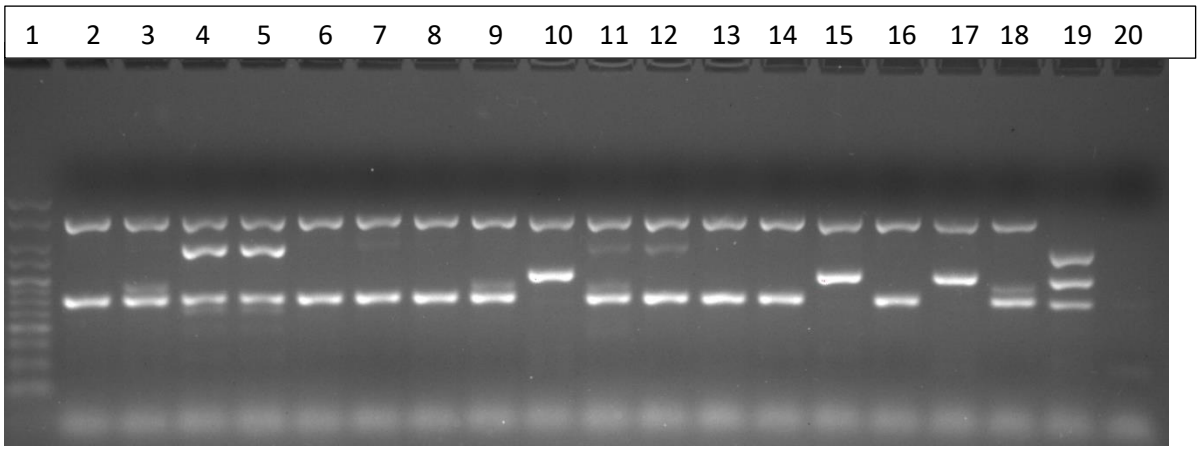
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	CW-37-S18	+	+	-	+	D+E
3	CW-47-A18	+	+	-	+	D+E
4	CW-56-A18	+	-	-	+	B1
5	CW-38-A18	+	-	-	+	B1
6	CW-14-S17	+	-	-	+	B1
7	CW-55-A18	+	-	-	+	B1
8	CW-53-A18	+	-	-	+	B1
9	CW-52-S18	+	-	-	+	B1
10	CW-1-S18	+	-	-	+	B1
11	CW-21-W18	+	-	-	+	B1
12	CW-4-S18	+	-	-	+	B1
13	CW-49-S17	+	+	-	+	D+E
14	CW-54-A18	+	+	-	+	D+E
15	CW-3-S17	+	+	-	+	D+E
16	CW-41-A18	+	-	+	-	A+C
17	CW-27-S17	+	-	-	+	B1
18	CW-25-A18	-	-	+	-	Clade I or II
19	*E. coli* ATCC25922	-	+	+	+	B2
20	dH_2O					



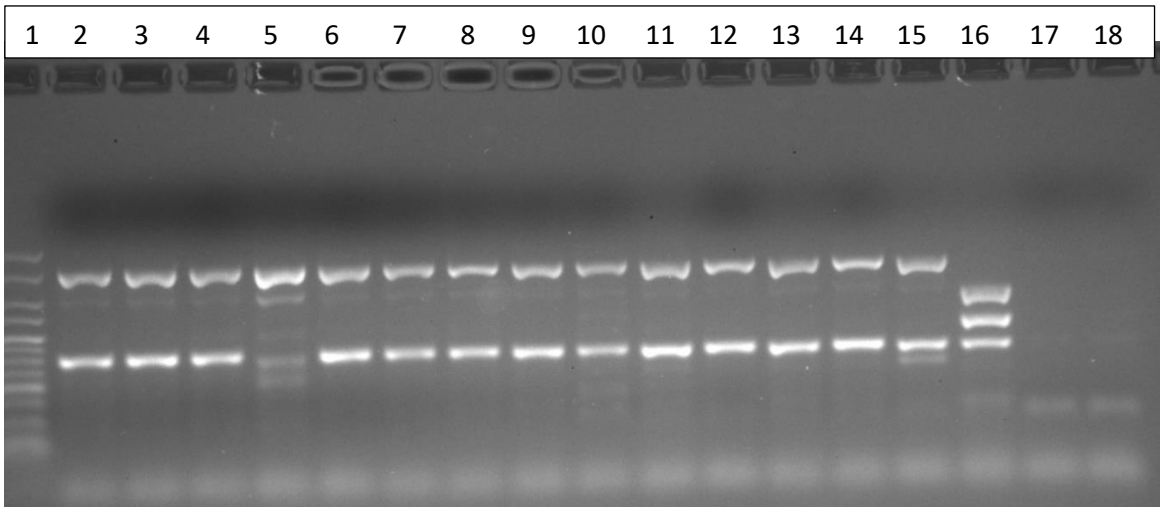
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	CW-23-S17	+	-	-	+	B1
3	CW-48-A18	+	-	-	+	B1
4	CW-51-A18	+	-	-	+	B1
5	CW-42-A18	+	-	+	-	A/C
6	CW-21-S18	+	-	-	+	B1
7	CW-59-A18	+	-	+	-	A/C
8	CW-31-S18	+	+	-	-	D/E
9	CW-28-S17	+	-	-	+	B1
10	CW-29-S17	+	-	-	+	B1
11	CW-49-A18	+	+	-	-	D/E
12	CW-62-A18	+	-	-	+	B1
13	CW-5-A18	+	-	-	+	B1
14	CW-38-A18	+	-	-	+	B1
15	CW-44-S17	+	-	-	+	B1
16	CW-57-A18	-	-	-	-	unknown
17	CW-7-A18	+	+	-	+	D/E
18	*E. coli* ATCC25922	-	+	+	+	B2
19	CW-22-A18					
20	dH_2O					



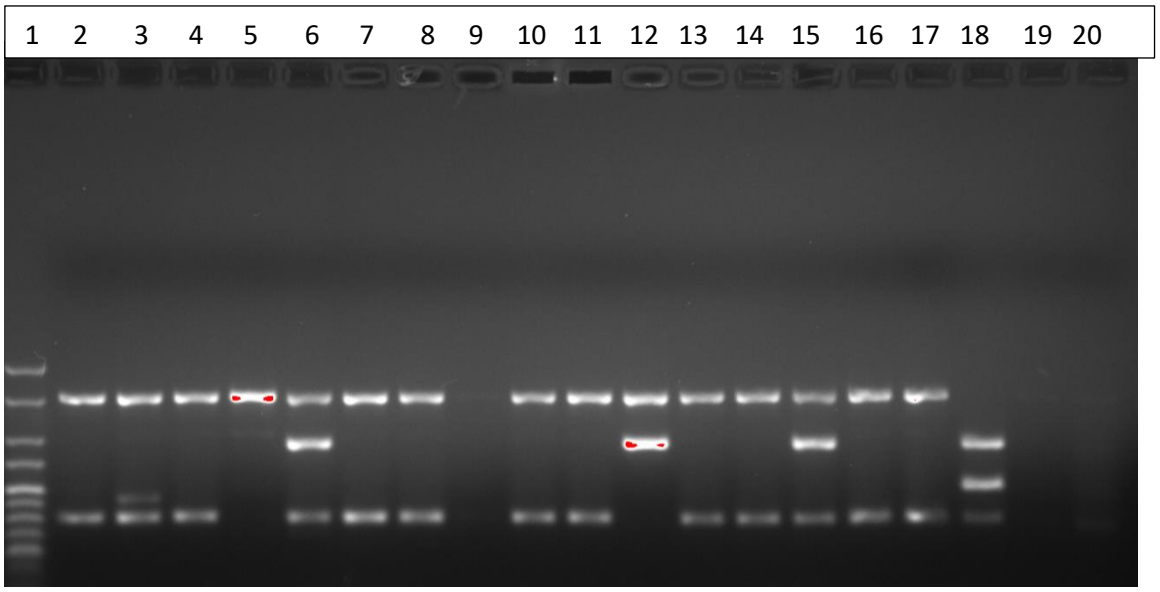
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	PF-63-W18	+	-	-	+	B1
3	PF-20-W18	+	+	-	-	D+E
4	MRD-23-W18	+	-	-	+	B1
5	MRD-2[1]-W18	+	-	-	+	B1
6	PF-21-W18	+	-	-	+	B1
7	PF-19-W18	+	-	-	+	B1
8	MRD-45-W18	+	-	-	+	B1
9	PF-24-W18	+	-	-	+	B1
10	MRD-3-W18	+	-	-	+	B1
11	MRD-34-W18	+	-	-	+	B1
12	MRD-3-W18	+	+	-	+	D+E
13	MRD-2-W18	+	-	-	+	B1
14	MRD-4-W18	+	-	-	+	B1
15	PF-32-W18	-	-	-	-	unknown
16	*E. coli* ATCC25922	-	+	+	+	B2
17	dH_2O					
18						
19						



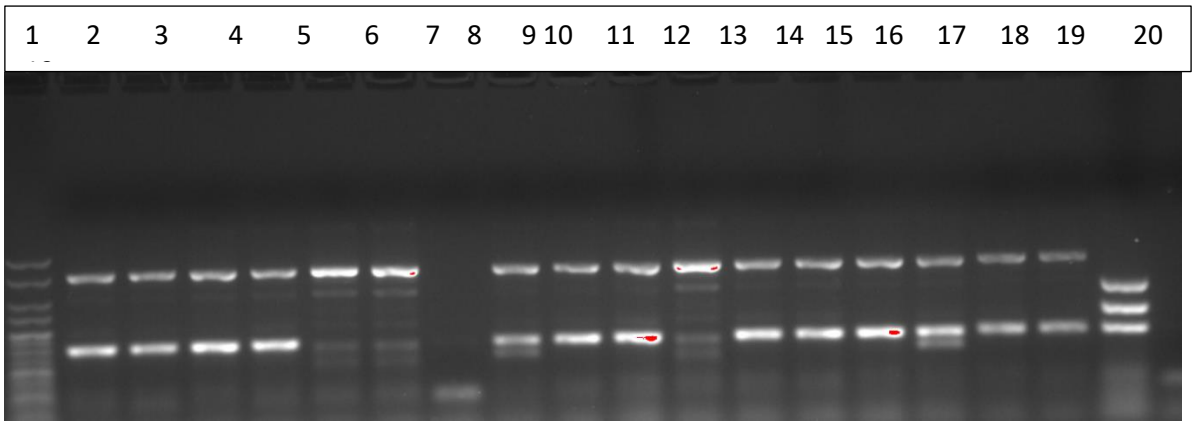
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	PF-31-W18	+	-	-	+	B1
3	PF-26-W18	+	-	-	+	B1
4	PF-23-W18	+	+	-	+	D/E
5	MRD-40-W18	+	+	-	+	D/E
6	MRD-24-W18	+	-	-	+	B1
7	MRD-12-W18	+	-	-	+	B1
8	PF-63-W18	+	-	-	+	B1
9	MRD-22-W18	+	-	-	+	B1
10	PF-25*-W18	+	-	+	+	UNKNOWN
11	PF-23*-W18	+	+	-	+	D+E
12	PF-18-W18	+	+	-	+	D+E
13	MRD-11-W18	+	-	-	+	B1
14	PF-28-W18	+	-	-	+	B1
15	PF-29-W18	+	-	-	-	A
16	PF-27-W18	+	-	-	+	B1
17	MRD-17-W18	+	-	+	-	A/C
18	MRD-34-W18	+	-	-	+	B1
19	*E. coli* ATCC25922	-	+	+	+	B2
20	dH_2O					



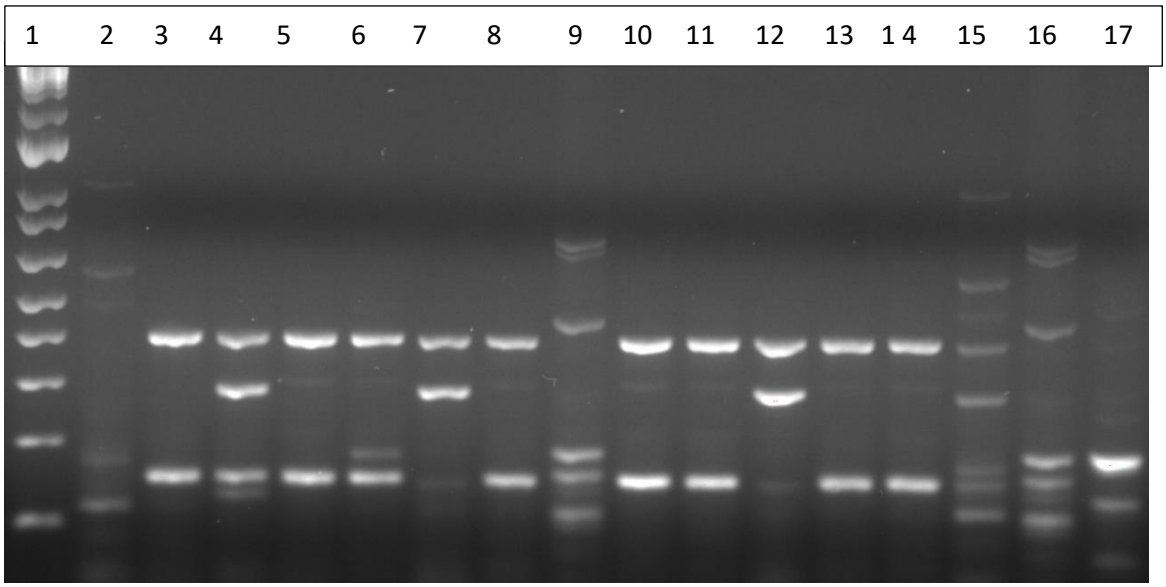
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	PF-5-A18	+	-	-	+	B1
3	PF-16-A18	+	-	-	+	B1
4	PF-58-A18	+	-	-	+	B1
5	PF-31-A18	+	-	-	-	A
6	PF-11-A18	+	+	-	+	D+E
7	PF-15-A18	+	-	-	+	B1
8	PF-4-A18	+	-	-	+	B1
9	PF-61-A18	+	-	-	+	B1
10	MRD-2-W18	+	-	-	+	B1
11	MRD-4-W18	+	-	-	+	B1
12	PF-23-A18	+	-	-	-	A
13	PF-60-A18	+	-	-	+	B1
14	PF-62-A18	+	-	-	+	B1
15	PF-3-A18	+	-	-	+	B1
16	PF-63-A18	+	-	-	+	B1
17	PF-14-A18	+	-	-	+	B1
18	*E. coli* ATCC25922	-	+	+	+	B2
19	dH_2O					
20	MM					



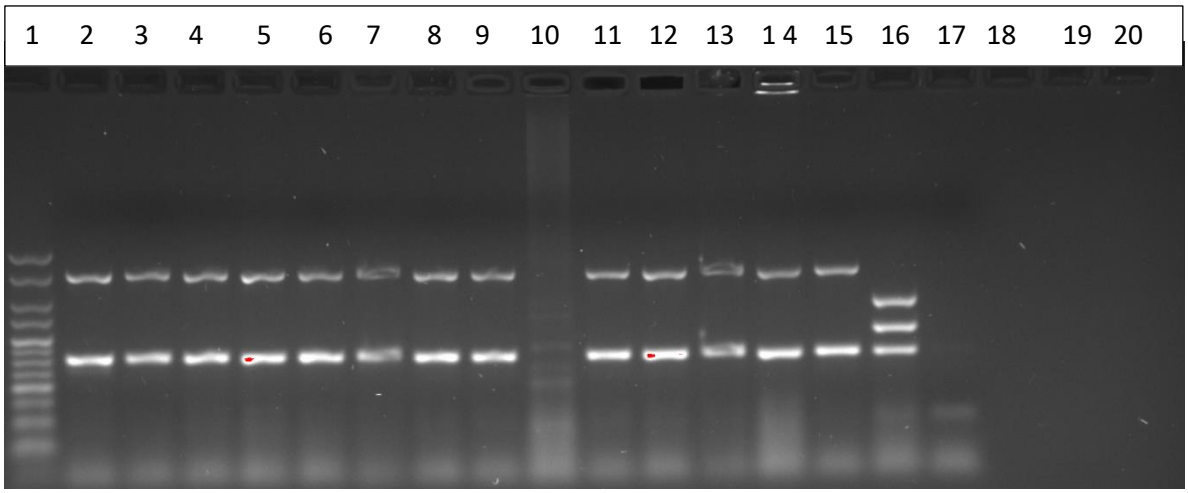
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	HyperV ladder					
2	PF-21-W18	+	-	-	+	B1
3	PF-20-W18	+	-	-	+	B1
4	PF-19-W18	+	-	-	+	B1
5	PF-22-W18	+	-	-	-	A
6	PF-18-W18	+	+	-	+	D+E
7	PF-25-W18	+	-	-	+	B1
8	PF-25*-W18	+	-	-	+	B1
9	PF-29-W18	+	-	-	-	A
10	PF-28-W18	+	-	-	+	B1
11	PF-30-W18	+	-	-	+	B1
12	PF-26-S18	+	+	-	-	D+E
13	PF-32-W18	+	-	-	+	B1
14	PF-31-W18	+	-	-	+	B1
15	PF-34-W 18	+	+	-	+	D+E
16	MRD-12-W18	+	-	-	+	B1
17	MRD-17-W18	+	-	-	+	B1
18	*E. coli* ATCC25922	-	+	+	+	B2
19	dH_2O					
20	MM					



S.No		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	HyperV ladder					
2	PF-34-A18	+	-	-	+	B1
3	PF-34-A18	+	-	-	+	B1
4	PF-3-A18	+	-	-	+	B1
5	PF-2-A18	+	-	-	+	B1
6	PF-19-A18	+	+	-	+	D+E
7	PF-22-A18	+	+	-	+	D+E
8	PF-25-A18	-	-	-	-	unknown
9	PF-24-A18	+	-	-	+	B1
10	PF-32-A18	+	-	-	+	B1
11	PF-13-A18	+	-	-	+	B1
12	PF-44-A18	+	-	-	-	A
13	PF-9-A18	+	-	-	+	B1
14	PF-58-A18	+	-	-	+	B1
15	PF-30-A18	+	-	-	+	B1
16	PF-21-A18	+	-	-	+	B1
17	PF-30-A18	+	-	-	+	B1
18	PF-46-A18	+	-	-	+	B1
19	*E. coli* ATCC25922	-	+	+	+	B2
20	dH_2O	-	-	-	-	



		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	1Kb+ladder					
2	PF-56-A18	-	-	-	-	unknown
3	PF-5A-18	+	-	-	+	B1
4	PF-11-A18	+	+	-	+	D+E
5	PF-4A-18	+	-	-	+	B1
6	PF-16A-18	+	-	-	+	B1
7	PF-50A-18	+	+	-	-	D+E
8	PF-60A-18	+	-	-	+	B1
9	PF-10A-18	476				III +IV+V
10	PF-63A-18	+	-	-	+	B1
11	PF-31A-18	+	-	-	+	B1
12	PF-61A-18	+	+	-	-	D+E
13	PF-62A-18	+	-	-	+	B1
14	PF-15A-18	+	-	-	+	B1
15	PF-34A-18	-	-	-	-	unknown
16	PF-23-A18	476				III +IV+V
17	PF-63^{+}-A18	-	-	+	-	I+II
18						
19						
20						



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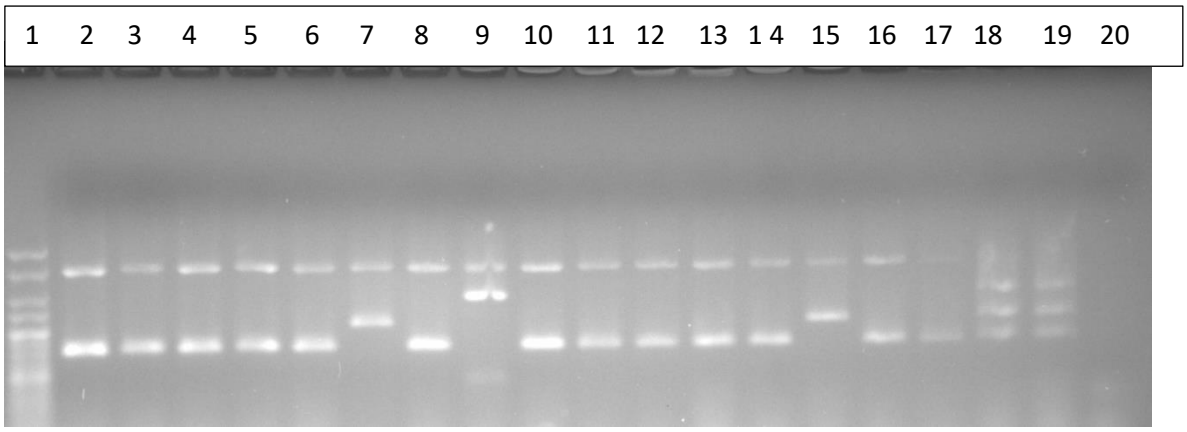
S.No		**Quadruplex genotype**				**Phylogroup**
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	TL-45-A18	+	-	-	+	B1
3	TL-50-A18	+	-	-	+	B1
4	TL-53-A18	+	-	-	+	B1
5	TL-54-A18	+	-	-	+	B1
6	TL-49-A18	+	-	-	+	B1
7	TL-46-A18	+	-	-	+	B1
8	TL-55-A18	+	-	-	+	B1
9	TL-41-A18	+	-	-	+	B1
10	TL-26-W18	-	-	-	-	unknown
11	TL-47-A18	+	-	-	+	B1
12	TL-48-A18	+	-	-	+	B1
13	TL-21-A18	+	-	-	+	B1
14	CW-61-A18	+	-	-	+	B1
15	CW-38-W18	+	-	-	+	B1
16	*E. coli* ATCC25922		+	+	+	B2
17	dH_2O	-	-	-	-	
18						
19						
20						



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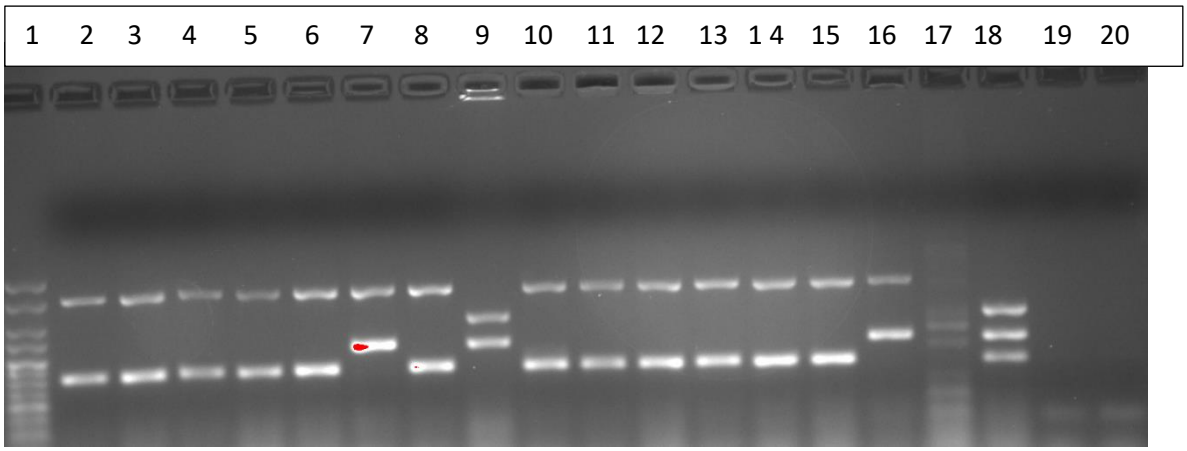
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	MRD-33-S17	+	-	-	+	B1
3	MRD-49-S17	+	-	-	+	B1
4	MRD-26-S17	+	-	-	+	B1
5	MRD-35-S17	+	-	-	+	B1
6	MRD-29-S17	+	-	+	-	B1
7	MRD-14-S17	+	-	+	-	A or C
8	MRD-24-S17	+	-	-	+	B1
9	MRD-38-S18	+	+	-	-	D or E
10	MRD-18-S17	+	-	-	+	B1
11	MRD-46-S17	+	-	-	+	B1
12	MRD-30-S17	+	-	-	+	B1
13	MRD-50-S17	+	-	-	+	B1
14	MRD-33-S17	+	-	-	+	B1
15	MRD-27-S17	+	-	+	-	A or C
16	MRD-17-S17	+	-	-	+	B1
17	MRD-40-S17	+	-	-	+	B1
18	MRD-40-S18	-	+	+	+	B2
19	*E. coli* ATCC25922	-	+	+	+	B2
20	DH20					



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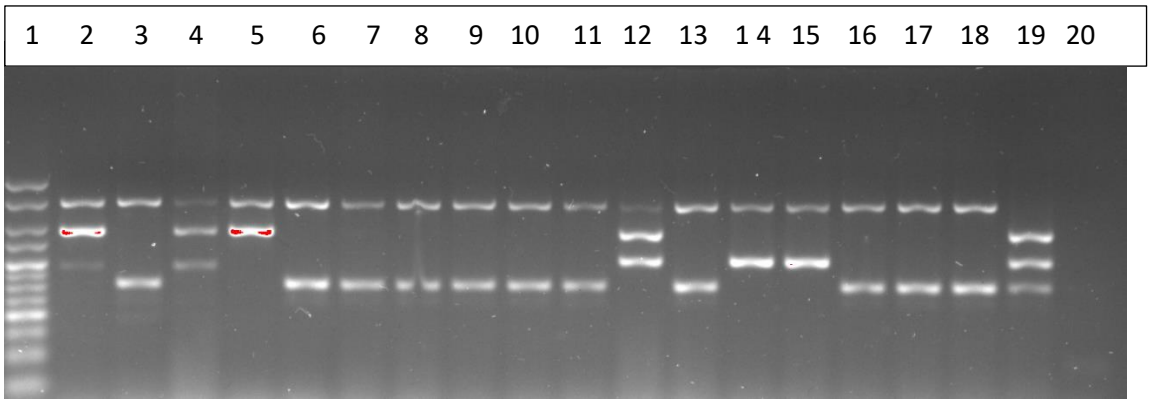
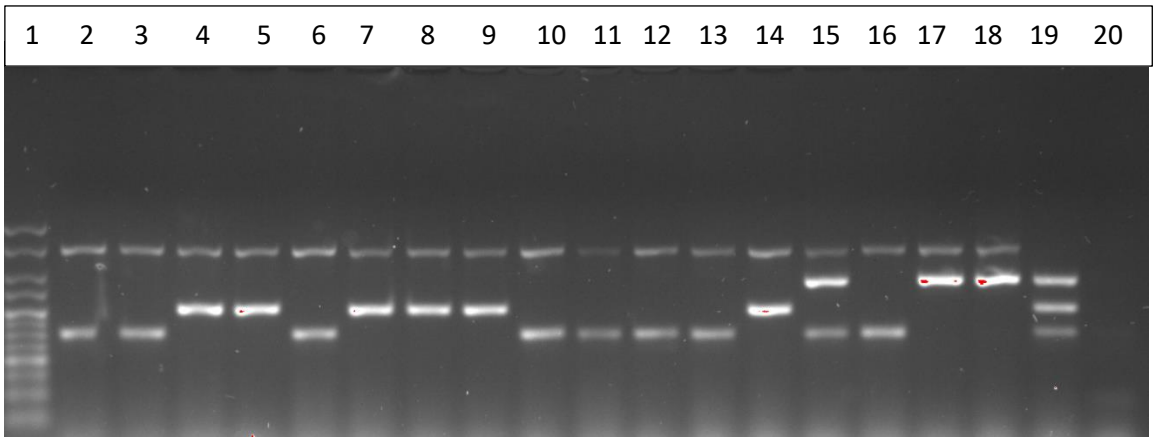
		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	MRD-4-S17	+	-	-	+	B1
3	MRD-13-S17	+	-	-	+	B1
4	MRD-33-S18	+	-	-	+	B1
5	MRD-42-S18	+	-	-	+	B1
6	MRD-3-S18	+	-	-	+	B1
7	MRD-2-S18	+	-	+	-	A or C
8	MRD-17-S18	+	-	-	+	B1
9	MRD-48-S18	-	+	+	-	B2
10	MRD-6-S18	+	-	-	+	B1
11	MRD-20-S18	+	-	-	+	B1
12	MRD-55-S17	+	-	-	+	B1
13	MRD-27-S18	+	-	-	+	B1
14	MRD-24-S18	+	-	-	+	B1
15	MRD-51-S18	+	-	-	+	B1
16	MRD-29-S17	+	-	+	-	A or C
17	MRD-38-S17	-	-	-	-	unknown
18	*E. coli* ATCC25922	-	+	+	+	B2
19	DH_20					
20	PCR MM					



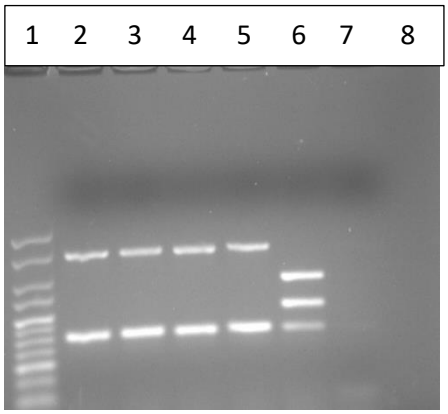
lincoln 2019-08-16_18h15m04sgel1.tif

S.No		**Quadruplex genotype**				**Phylogroup**
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	MRD-15-A18	+	+	-	-	D+E
3	MRD-29-A18	+	-	-	+	B1
4	MRD-26-A18	+	+	+	-	E or CLAD1
5	MRD-27-A18	+	+	-	-	D +E
6	MRD-22-A18	+	-	-	+	B1
7	MRD-58-A18	+	-	-	+	B1
8	MRD-28-A18	+	-	-	+	B1
9	PF-23-S17	+	-	-	+	B1
10	PF-40-S17	+	-	-	+	B1
11	PF-21-S17	+	-	-	+	B1
12	PF-42-S17	+	+	+	-	E or CLAD1
13	PF-50-S17	+	-	-	+	B1
14	PF-34*-S17	+	-	+	-	A+C
15	PF-34-S17	+	-	+	-	A+C
16	PF-35*-S17	+	-	-	+	B1
17	PF-35-S17	+	-	-	+	B1
18	PF-35-S17	+	-	-	+	B1
19	*E. coli* ATCC25922	-	+	+	+	B2
20	DH2O					



lincoln 2019-08-16_18h24m40sgel2.tif

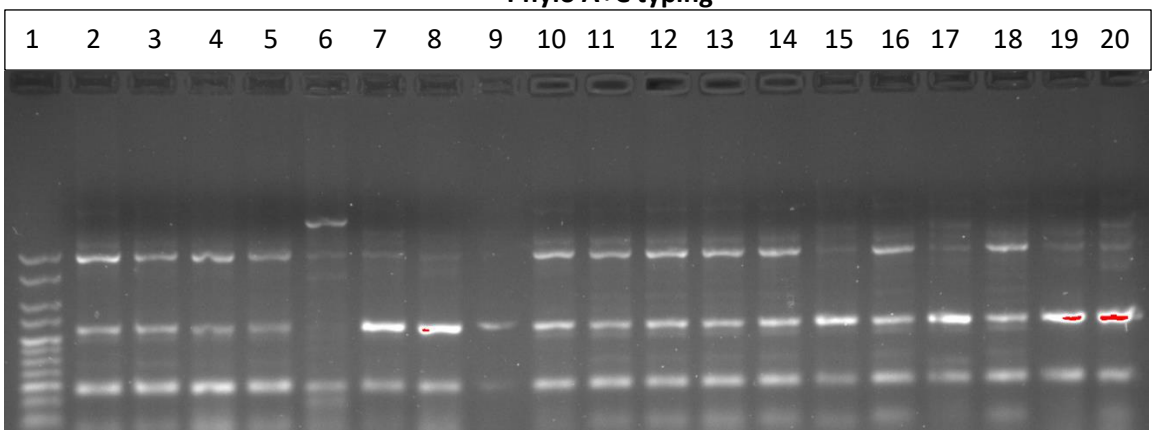
		Quadruplex genotype				**Phylogroup**
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	PF-44-S17	+	-	-	+	B1
3	PF-22-S17	+	-	-	+	B1
4	PF-19-S17	+	-	+	-	A+C
5	PF-18-S17	+	-	+	-	A+C
6	PF-16-S17	+	-	-	+	B1
7	PF-33-S17	+	-	+	-	A+C
8	PF-26-S17	+	-	+	-	A+C
9	PF-24-S17	+	-	+	-	A+C
10	PF-23-S17	+	-	-	+	B1
11	PF-15-S17	+	-	-	+	B1
12	PF-13-S17	+	-	-	+	B1
13	PF-35-S17	+	-	-	+	B1
14	PF-5-S17	+	-	+	-	A+C
15	PF-20-S17	+	+	-	+	D+E
16	PF-16-S17	+	-	-	+	B1
17	PF-41-S17	+	+	-	-	D+E
18	PF-4-S17	+	+	-	-	D+E
19	*E. coli* E. COLI ATCC25922	-	+	+	+	B2
20	DH2O					



lincoln 2019-08-16_18h18m05sgel3.tif

		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder	+	-	-	+	
2	MRD-19-A18	+	-	-	+	B1
3	MRD-34-A18	+	-	-	+	B1
4	MRD-32-A18	+	-	-	+	B1
5	MRD-25-A18	+	-	-	+	B1
6	*E. coli* ATCC25922	-	+	+	+	B2
7	DH2O					
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						

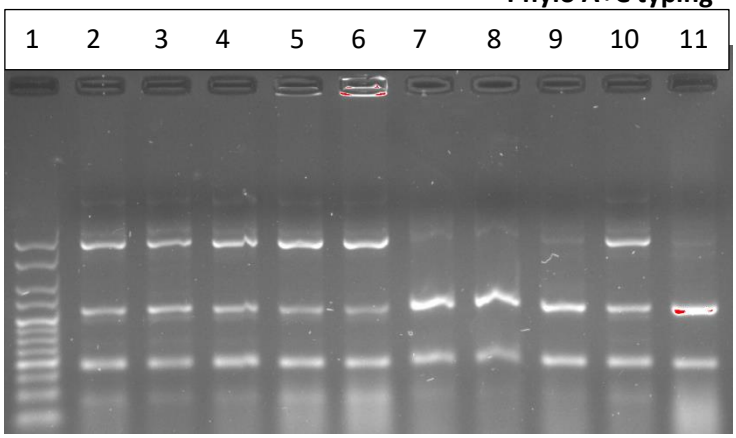
Phylo A+C typing



lincoln 2019-09-04_15h34m30s phylo C gel1.tif

S.No		**Quadruplex genotype**				**Phylogroup**
Lane number	**Strain Name**			***trpBA***	***trpAgpC***	
1	Hyper V ladder			+	+	
2	MRD-2-S18			+	+	C
3	MRD-47-S18			+	+	C
4	MRD-44-S17			+	+	C
5	MRD-27-S17			+	+	C
6	TL-18-A18			+	-	A
7	CW-19-S18			+	+	C
8	CW-26-A18			+	+	C
9	CW-7-S18			+	+	C
10	CW-6-A18			+	+	C
11	PF-30-S17			+	+	C
12	PF-26-S17			+	+	C
13	PF-9-S17			+	+	C
14	PF-8-S17			+	+	C
15	PF-56-S18			+	+	C
16	PF-6-S17			+	+	C
17	PF-9-S18			+	+	C
18	PF-5-S17			+	+	C
19	PF-25*-W18			+	+	C
20	PF-33-S17			+	+	C

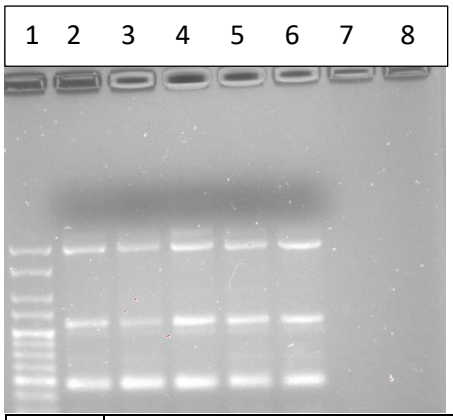
Phylo A+C typing



lincoln 2019-09-04_15h46m17s phyloC gel2.tif

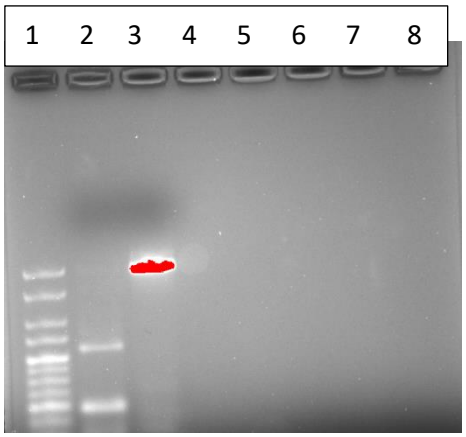
		A+C typing				Phylogroup
Lane number	**Strain Name**			***trpBA***	***trpAgpC***	
1	Hyper V ladder					
2	PF-27-S17			+	+	C
3	PF-13-W18			+	+	C
4	PF-38-S17			+	+	C
5	PF-51-A18			+	+	C
6	PF-54-A18			+	+	C
7	CW-15-S18			+	+	C
8	CW-16-S18			+	+	C
9	CW-14-S18			+	+	C
10	CW-26-W18			+	+	C
11	CW-39-A18			+	+	C

Phylo A+C typing



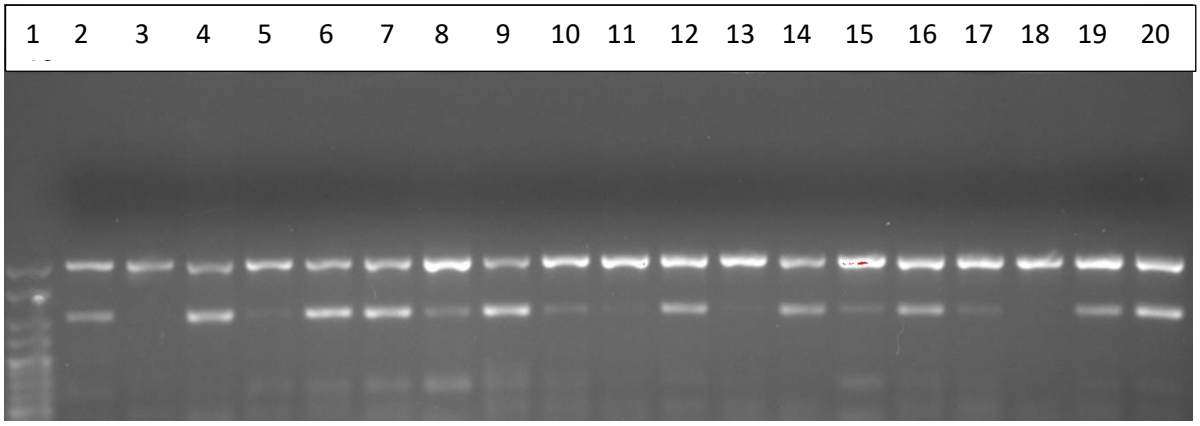
S.No		A+C typing				Phylogroup
Lane number	**Strain Name**			***trpBA***	***trpAgpC***	
1	Hyper V ladder					
2	PF-19-S17			+	+	C
3	PF-24-S17			+	+	C
4	PF-26-S17			+	+	C
5	PF-34-S17			+	+	C
6	PF-33-S17			+	+	C
7						
8						

Phylo A+C typing



lincoln 2019-09-05_18h13m40s TL14S17A+C.tif

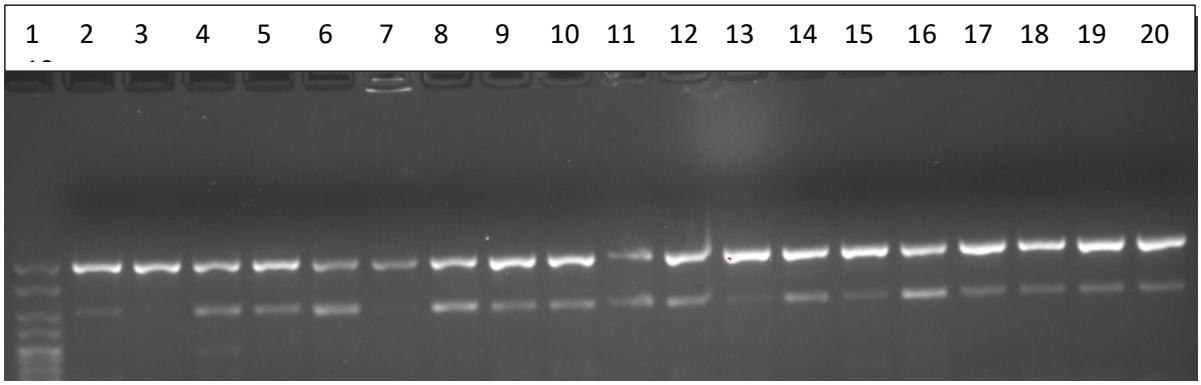
S.No		**A+C typing**				**Phylogroup**
Lane number	**Strain Name**			***trpBA***	***trpAgpC***	
1	Hyper V ladder					
2	TL-14-S17			-	+	C
3	TL-14-S17			+	-	
4						
5						
6						
7						
8						



lincoln 2019-09-06_15h33m26s phyloD+E.tif

		D+E genotyping				Phylogroup
Lane number	**Strain Name**			***trpBA***	***trpAgpE***	
1	Hyper V ladder					
2	CW-10-S18			+	+	E
3	CW-43-W18			+	-	D
4	CW-25-S18			+	+	E
5	CW-39-W18			+	+	E
6	CW-31-S18			+	+	E
7	CW-20-S18			+	+	E
8	CW-33-S18			+	+	E
9	CW-30-S18			+	+	E
10	CW-36-W18			+	+	E
11	CW-1-A18			+	+	E
12	CW-44-A18			+	+	E
13	CW-34-W18			+	+	E
14	CW-44-S18			+	+	E
15	CW-49-S17			+	+	E
16	CW-33-S18			+	+	E
17	CW-48-W18			+	+	E
18	CW-43-W18			+	-	D
19	CW-27-S18			+	+	E
20	CW-16-A18			+	+	E

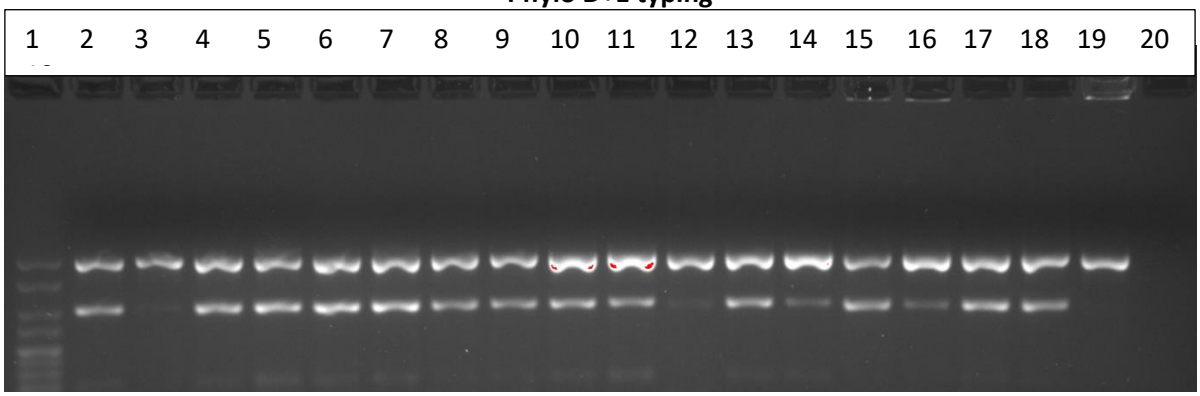
Phylo D+E typing



lincoln 2019-09-06_15h40m06sphyloEgel2.tif

		Quadruplex genotype				Phylogroup
Lane number	**Strain Name**			***trpBA***	***trpAgpE***	
1	Hyper V ladder					
2	CW-23-A18			+	+	E
3	CW-41-W18			+	-	D
4	CW-32-S18			+	+	E
5	CW-29-W18			+	+	E
6	CW-39-S18			+	-	D
7	CW-35-S18			+	+	E
8	CW-27-W18			+	+	E
9	PF-41-S18			+	+	E
10	PF-18-W18			+	+	E
11	PF-41-S17			+	+	E
12	PF-55-A18			+	+	E
13	PF-11-A18			+	+	E
14	PF-20-S17			+	+	E
15	PF-29-S17			+	+	E
16	PF-42-S17			+	+	E
17	PF-29-W18			+	+	E
18	PF-43-A18			+	-	D
19	PF-26-S18			+	+	E
20	PF-23-W18			+	+	E

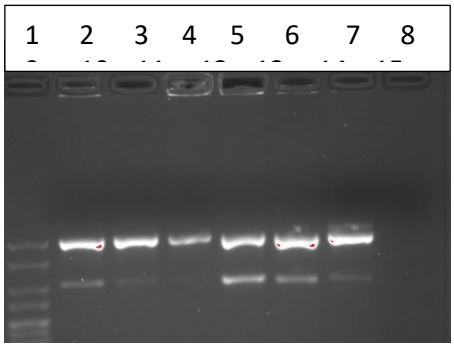
Phylo D+E typing



lincoln 2019-09-07_15h25m57s phylo E gel1.tif

		D+E typing				Phylogroup
Lane number	**Strain Name**			***trpBA***	***trpAgpE***	
1	Hyper V ladder					
2	MRD-30-S18			+	+	E
3	MRD-27-A18			+	+	D
4	MRD-41-S18			+	+	E
5	MRD-53-S17			+	+	E
6	CW-18-A18			+	+	E
7	TL-42-S17			+	+	E
8	MRD-38-S18			+	+	E
9	MRD-31-S18			+	+	E
10	TL-51-S18			+	+	E
11	PF-29-S18			+	+	E
12	MRD-44-S17			+	+	E
13	MRD-47-W18			+	+	E
14	MRD-46-W18			+	+	E
15	MRD-8-S17			+	+	E
16	MRD-40*-S17			+	+	E
17	MRD-25-W18			+	+	E
18	MRD-26-W18			+	-	D
19	TL-45-S18			+	+	E
20	dH_20			-	-	

Phylo D+E typing

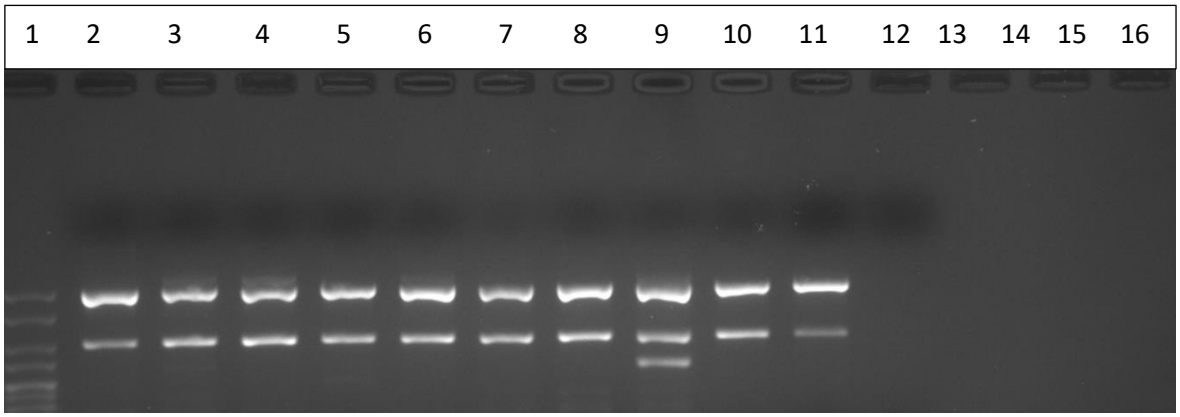


lincoln 2019-09-07_15h29m33s phyloE gel2.tif

S.No		**Quadruplex genotype**				**Phylogroup**
Lane number	**Strain Name**			***trpBA***	***trpAgpE***	
1	Hyper V ladder					
2	TL-1-S17			+	+	E
3	TL-40-S17			+	+	E
4	TL-32-S17			+	+	D
5	TL-32*-S17			+	+	E
6	PF-57-S17			+	+	E
7	PF-34-S17			+	+	E
	dH_20			-	-	

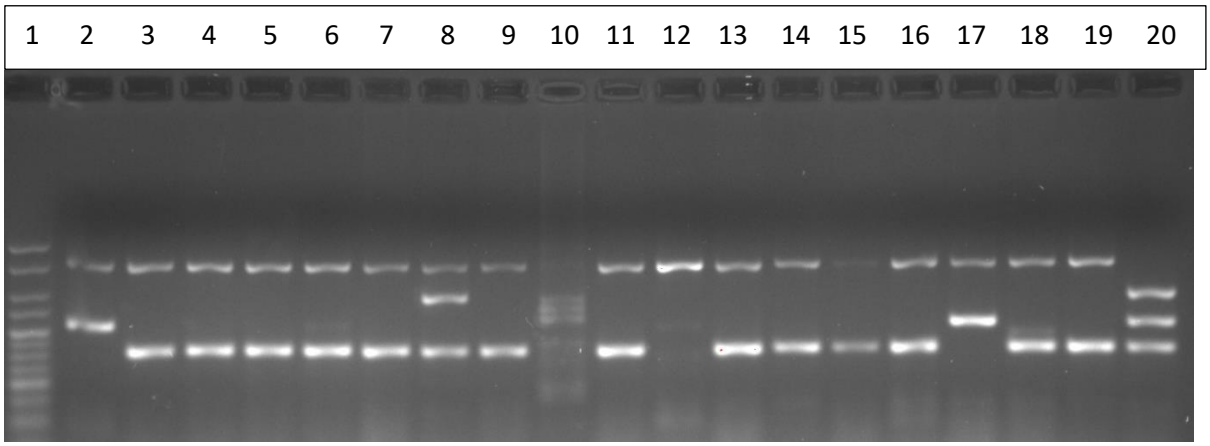
*** A LARGE SECOND COLONY FROM SAME STREAKED PLATE WITH MORE DNA TEMPLATE**

D+E typing



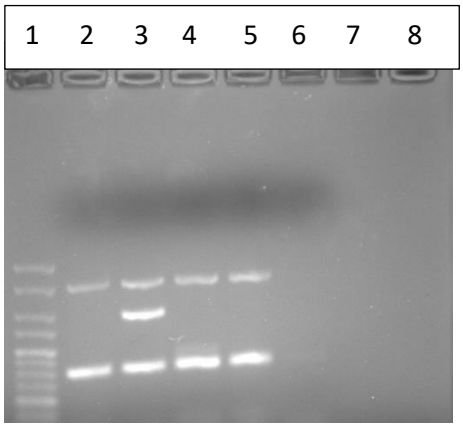
lincoln 2019-09-11_15h04m46s phyloE.tif

		D+E typing				Phylogroup
Lane number	**Strain Name**			***trpBA***	***trpAgpE***	
1	Hyper V ladder			+	+	E
2	CW-8-A18			+	+	E
3	CW-14-A18			+	+	E
4	CW-28-W18			+	+	E
5	PF-40-A18			+	+	E
6	CW-14-A18			+	+	E
7	CW-37-A18			+	+	E
8	CW-35-W18			+	+	E
9	PF-37-S17			+	+	E
10	PF-12-A18			+	+	E
11	CW-26-S18			+	+	E
12	DH_20					
13						
14						
15						
16						
17						
18						
19						
20						



lincoln 2019-09-11_17h15m52s multiplex gel1.tif

		Multiplex genotyping				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	CW-41-S17	+	-	+	-	A+C
3	CW-33-S17	+	-	-	+	B1
4	CW-5-S17	+	-	-	+	B1
5	CW-10-W18	+	-	-	+	B1
6	CW-4-S17	+	-	-	+	B1
7	CW-17-S17	+	-	-	+	B1
8	CW-33-W18	+	+	-	+	D+E
9	CW-29-S17	+	-	-	+	B1
10	CW-19-W18	+	-	-	-	A
11	CW-18-S17	+	-	-	+	B1
12	CW-44-W18	+	-	-	-	A
13	CW-27-S17	+	-	-	+	B1
14	CW-1-W18	+	-	-	+	B1
15	CW-25-S17	+	-	-	+	B1
16	CW-20-S17	+	-	-	+	B1
17	CW-51-S17	+	-	+	-	A+C
18	CW-20-A18	+	-	-	+	B1
19	CW-46-W18	+	-	-	+	B1
20	*E. coli* ATCC25922	+	-	-	+	B2



lincoln 2019-09-11_17h24m16s multiplexgel2.tif

		Multiplex genotyping				**Phylogroup**
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	CW-13-S17	+	-	-	+	B1
3	CW-3-S17	+	+	-	+	D+E
4	CW-2-S17	+	-	-	+	B1
5	CW-51-W18	+	-	-	+	B1
6						
7						
8						
9						
10						
11						
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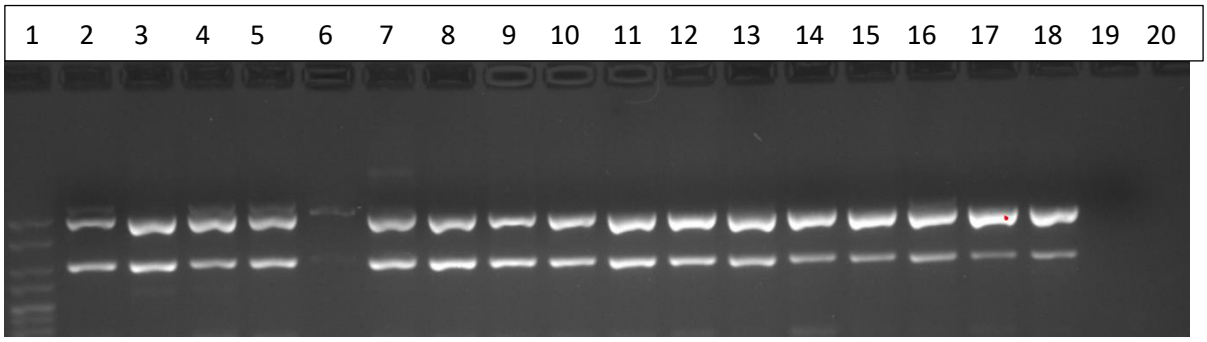
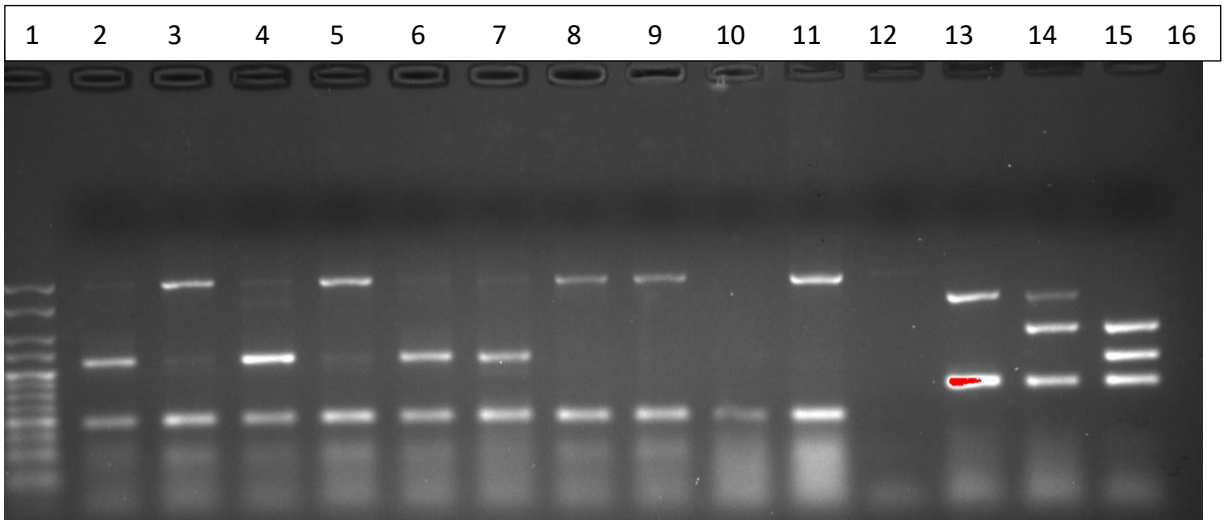
15						
16						
17						
18						
19						
20						



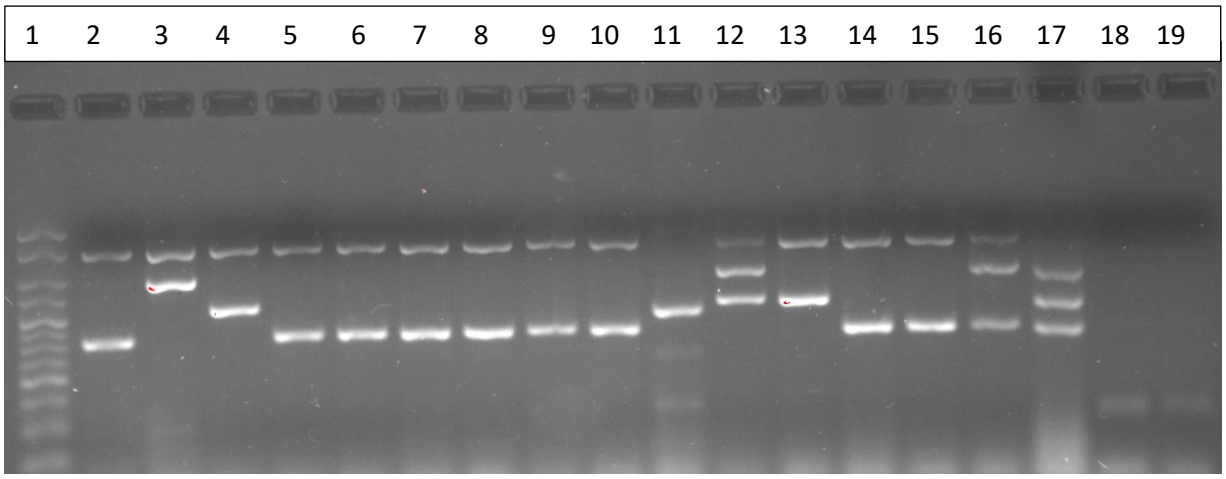
lincoln 2019-09-12_16h03m03sphyloE.tif

		D+E typing				Phylogroup
Lane number	**Strain Name**			***trpBA***	***trpAgpE***	
1	Hyper V ladder					
2	PF-45-W18			+	+	E
3	PF-32-S17			+	+	E
4	PF-41-S18			+	+	E
5	PF-45*-W18			+	+	E
6	PF-29-S18			+	+	E
7	PF-20-S17			+	+	E
8	PF-41-S17			+	+	E
9	PF-48-S18			+	+	E
10	PF-57-S18			+	+	E
11	PF-61-A18			+	+	E
12	PF-50-A18			+	+	E
13	MRD-1-S17			+	+	E
14	MRD-31-W18			+	+	E
15	MRD-40-W18			+	+	E
16	TL-51-S18			+	+	E
17	CW-36-A18			+	+	E
18	PF-57-A18			+	+	E
19	DH_20					
20	blank					



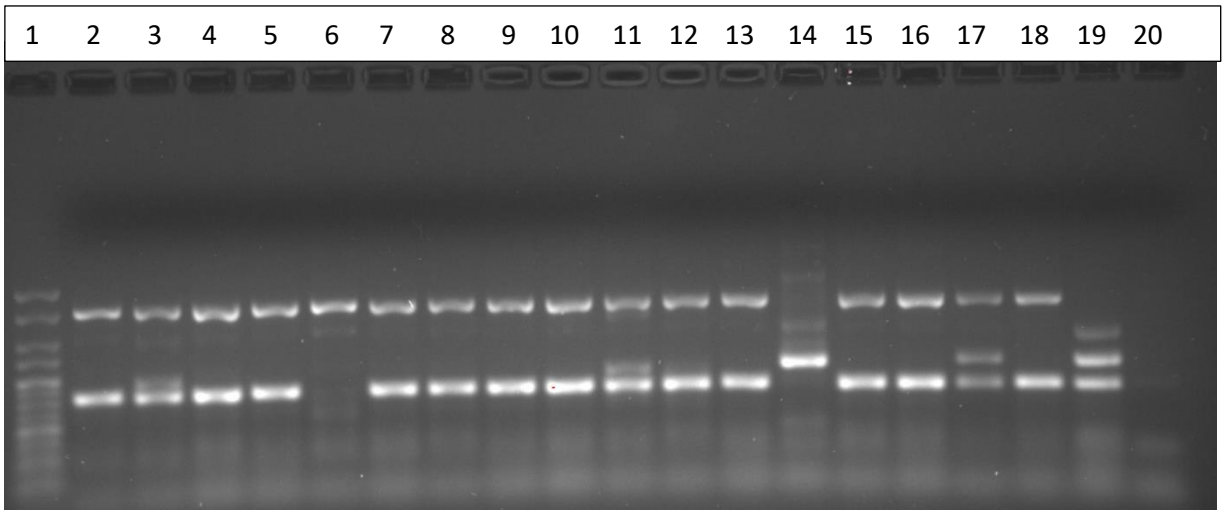
lincoln 2019-09-12_16h33m46sphyloC.tif

		A+C typing				Phylogroup
Lane number	**Strain Name**			***trpBA***	***trpAgpC***	
1	Hyper V ladder					
2	TL-11-S17			+	+	C
3	TL-5-S17			+	+	C
4	MRD-27-S17			+	+	C
5	PF-28-S17			+	+	C
6	PF-29-W18			+	+	C
7	CW-39-A18			+	+	C
8	CW-10-S17			+	-	A
9	CW-31-A18			+	-	A
10	CW-39*-A18			-	-	CONTAMINANT
11	PF-18-S17			-	-	A
12	DH_20			-	-	
MULTIPLEX GENOTYPING						
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	CW-20-A18	+	-	-	+	B1
2	CW-50-W18	+	+	-	+	D+E
3	*E. coli* ATCC25922	-	+	+	+	B2



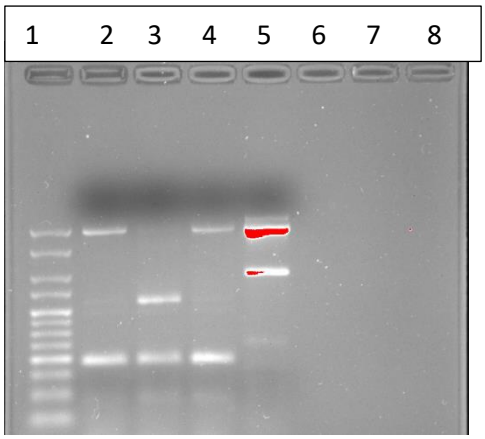
lincoln 2019-09-14_16h27m45smultiplex.tif

		Multiplex genotyping				Phylogroup
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	CW-47-A18	+	-	-	+	B1
3	CW-49-A18	+	+	-	-	D+E
4	CW-41-A18	+	-	+	-	A+C
5	CW-11-A18	+	-	-	+	B1
6	CW-5-A18	+	-	-	+	B1
7	CW-51-A18	+	-	-	+	B1
8	CW-52-A18	+	-	-	+	B1
9	CW-34-A18	+	-	-	+	B1
10	CW-22-A18	+	-	-	+	B1
11	CW-25-A18	-	-	+	-	Clade I or II
12	CW-35-A18	+	+	+	-	E or clade I
13	CW-42-A18	+	-	+	-	A+C
*14	CW-38-A18	+	-	-	+	B1
15	CW-38-A18	+	-	-	+	B1
16	CW-7-A18	+	+	-	+	D+E
17	*E. coli* ATCC25922	-	+	+	+	B2
18	DH20					
19	PRIMER MIX					
20						



lincoln 2019-09-14_16h27m45smultiplex.tif

		Multiplex genotyping				**Phylogroup**
Lane number	**Strain Name**	***ArpA***	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	MRD-23-W18	+	-	-	+	B1
3	MRD-32-W18	+	-	-	+	B1
4	MRD-15-S17	+	-	-	+	B1
5	MRD-4-S17	+	-	-	+	B1
6	MRD-20-W18	+	-	-	-	A
7	MRD-21-A18	+	-	-	+	B1
8	MRD-15-S17	+	-	-	+	B1
9	MRD-24-S17	+	-	-	+	B1
10	TL-35-A18	+	-	-	+	B1
11	TL-2-A18	+	-	-	+	B1
12	TL-25-S17	+	-	-	+	B1
13	TL-36-S17	+	-	-	+	B1
14	TL-56-A18	-	-	+	-	Clade I or II
15	TL-44-A18	+	-	-	+	B1
16	TL-23-A18	+	-	-	+	B1
17	TL-8-S17	+	-	+	+	unknown
18	PF-47-S17	+	-	-	+	B1
19	*E.coli* E. COLI ATCC25922	-	+	+	+	B2
20	DH_20					



S.No		PHYLO C TYPING				Phylogroup
Lane number	**Strain Name**			***trpBA***	***trpAgpC***	
1	Hyper V ladder					
2	PF-17-S17			+	+	C
3	CW-12-A17			+	+	C
4	PF-9-A18			+	+	C
				trpBA	***trpAgpE***	
5	PF-22-A18			+	+	E
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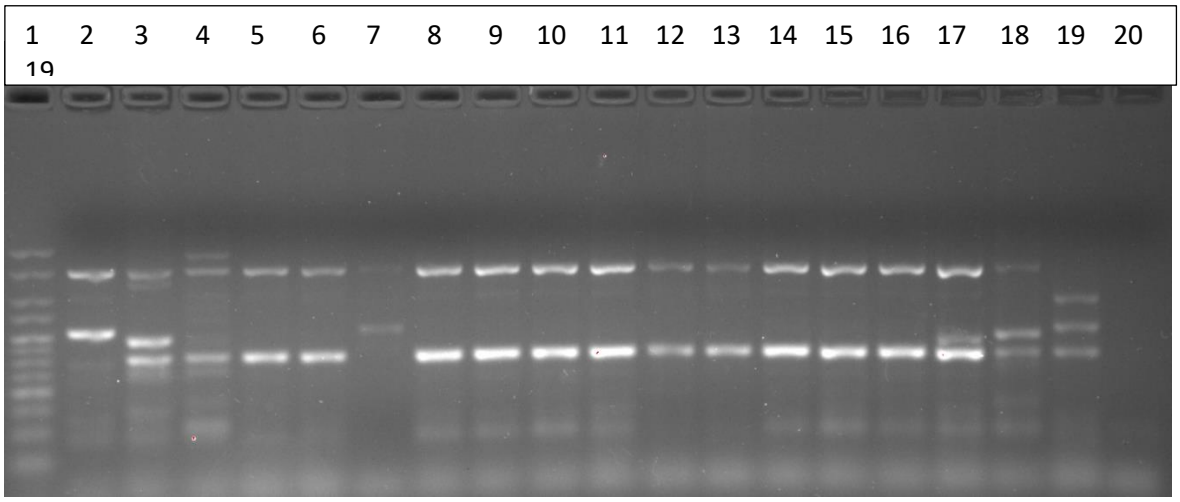
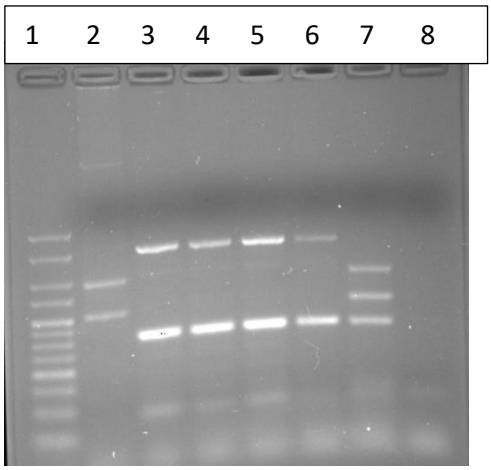


lincoln 2019-09-17_17h19m22smultiplex gel1.tif

S.No	Strain Name	Multiplex genotyping				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	PF-45-W18	+	+	+	-	E or clade I
3	PF-37-W18	+	-	-	+	B1
4	PF-12-W18	+	-	-	+	B1
5	TL-29-S17	+	-	-	+	B1
6	TL-31-S17	+	-	-	+	B1
7	TL-2-S17	+	-	+	-	A+C
8	PF-38-S18	+	-	-	+	B1
9	PF-17-S17	+	-	-	+	B1
10	PF-51-S17	+	-	-	+	B1
11	PF-38-S18	+	-	-	+	B1
12	PF-12-S17	+	-	-	+	B1
13	PF-10-S17	+	-	-	+	B1
14	PF-3-S17	+	-	-	+	B1
15	PF-11-S17	+	-	-	+	B1
16	PF-4-S17	+	-	-	+	B1
17	MRD-36-A18	+	-	-	+	B1
18	MRD-39-A18	+	-	-	+	B1
19	*E. coli* E. COLI ATCC25922	-	+	+	+	B2
20	DH_20	-	-	-	-	



lincoln 2019-09-17_17h21m41smultiplex gel2.tif

S.No	Strain Name	Multiplex genotyping				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	MRD-54-A18	-	+	+	-	B2
3	MRD-50-S18	+	-	-	+	B1
4	CW-2-S17	+	-	-	+	B1
5	CW-9-S17	+	-	-	+	B1
6	CW-47-S18	+	-	-	+	B1
7	*E. coli* E. COLI ATCC25922	-	+	+	+	B2
8	DH20	-	-	-	-	
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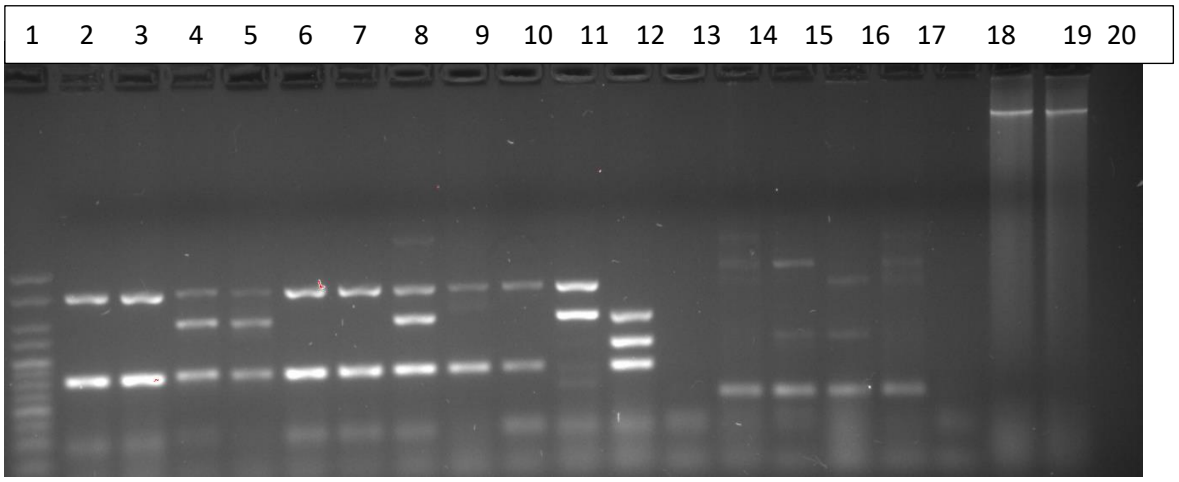
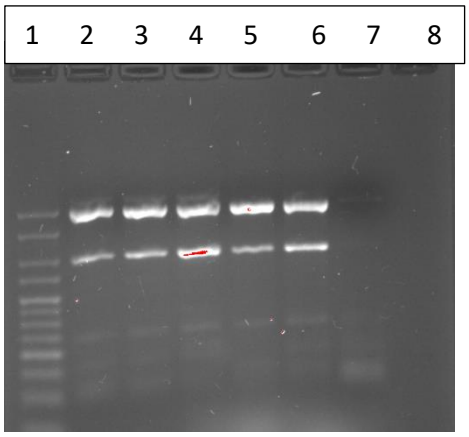


lincoln 2019-09-19_15h28m11Multiplex and A+C.tif

S.No	Strain Name	Multiplex genotyping				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	CW-24-S17	+	-	-	+	B1
3	CW-5-S17	+	-	-	+	B1
4	CW-35-W18	+	+	-	+	D+E
5	CW-49-W18	+	+	-	+	D+E
6	CW-51-A18	+	-	-	+	B1
7	CW-38-W18	+	-	-	+	B1
8	CW-8-S18	+	+	-	+	D+E
9	MRD-42-A18	+	-	-	+	B1
10	MRD-57-A18	+	-	-	+	B1
11	MRD-5-A18	+	+	-	+	D+E
12	*E. coli* ATCC25922	-	+	+	+	B2
13	DH20					
	A+C GENOTYPING					
				trpBA	***trpAgpC***	
14	TL-2-S17			+	-	A
15	TL-5-S17			+	+	C
16	TL-39-S17			+	+	C
17	CW-41-S17			+	-	A
18	DH20					
19	VICKY PLASMID					
20	VICKY PLASMID					



lincoln 2019-09-20_16h37m16sphyloE.tif

S.No	Strain Name	Phylo E genotyping				Phylogroup
				trpBA	***trpAgpE***	
1	Hyper V ladder					
2	CW-33-W18			+	+	E
3	CW-49-W18			+	+	E
4	CW-8-S18			+	+	E
5	CW-50-W18			+	+	E
6	MRD-5-A18			+	+	E
7	DH20					
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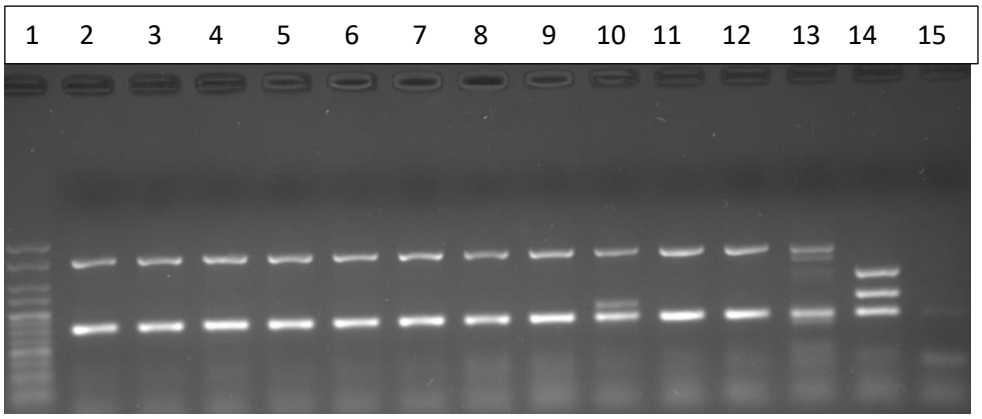
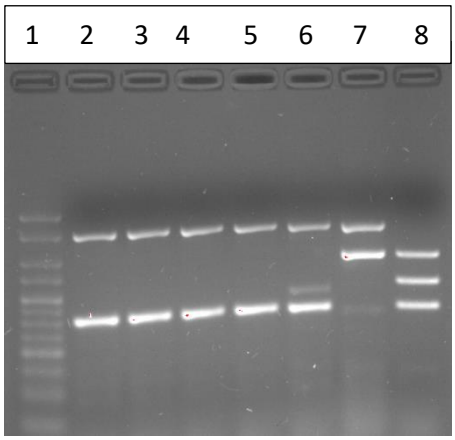


lincoln 2019-09-25_14h17m59smultiplex.tif

S.No	Strain Name	Multiplex genotyping				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	TL-7-S18	+	-	-	+	B1
3	TL-9-S18	+	-	-	+	B1
4	TL-10-S18	+	-	-	+	B1
5	TL-6-S18	+	-	-	+	B1
6	TL-12-S18	+	-	-	+	B1
7	TL-18-S18	+	-	-	+	B1
8	PF-25-W18	+	-	-	+	B1
9	PF-26-W18	+	-	-	+	B1
10	PF-52-W18	+	-	-	+	B1
11	PF-37-W18	+	-	-	+	B1
12	PF-42-W18	+	-	-	+	B1
13	PF-39-W18	+	-	-	+	B1
14	*E. coli* ATCC25922	-	+	+	+	B2
15	DW	-	-	-	-	
16						
17						
18						
19						
20						



S.No	Strain Name	Multiplex genotyping				Phylogroup
		ArpA	*chu A*	*yja A*	*TspE4C2*	
1	Hyper V ladder					
2	MRD-3-W18	+	-	-	+	B1
3	MRD-21-W18	+	-	-	+	B1
4	MRD-33-W18	+	-	-	+	B1
5	MRD-13-A18	+	-	-	+	B1
6	MRD-14-A18	+	-	-	+	B1
7	MRD-15-A18	+	+	-	+	D+E
8	*E. coli* ATCC25922	-	+	+	+	B2
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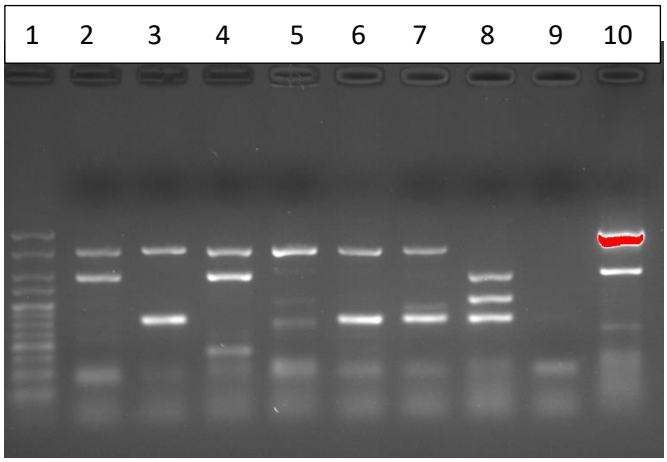

lincoln 2019-09-27_15h26m46smultilex &D+E.tif

S.No	Strain Name	Multiplex genotyping				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	Hyper V ladder					
2	TL-39-S18	+	+	-	-	D+E
3	MRD-40-S17	+	-	-	+	B1
4	MRD-38-S18	+	+	-	-	D+E
5	PF-55-W18	+	-	-	-	A
6	MRD-51-S17	+	-	-	+	B1
7	PF-52-W18	+	-	-	+	B1
8	*E. coli* ATCC25922	-	+	+	+	B2
9	DW					
	D+E TYPING			***trpBA***	***trpAgpE***	**PHYLOGROUP**
11	MRD-15-A18			+	+	E
12						
13						
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19						
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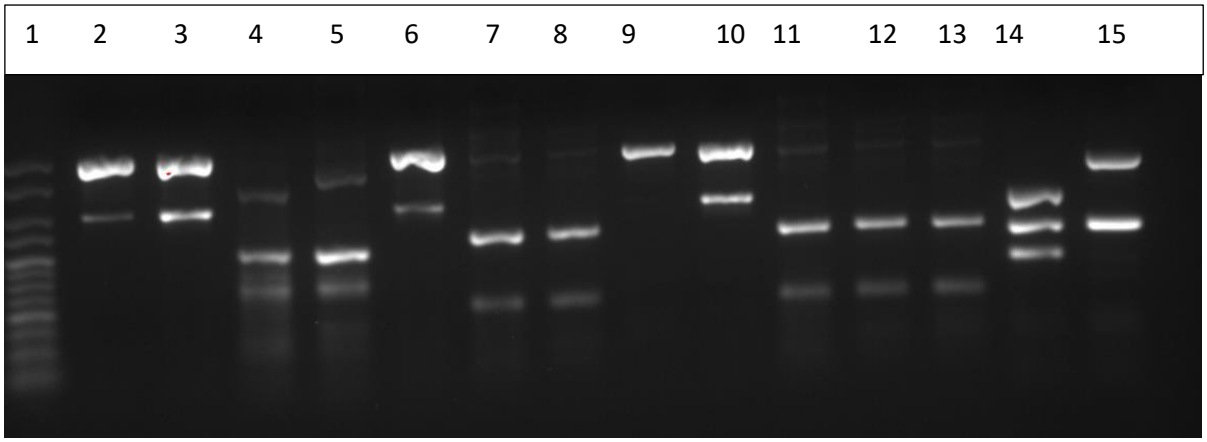
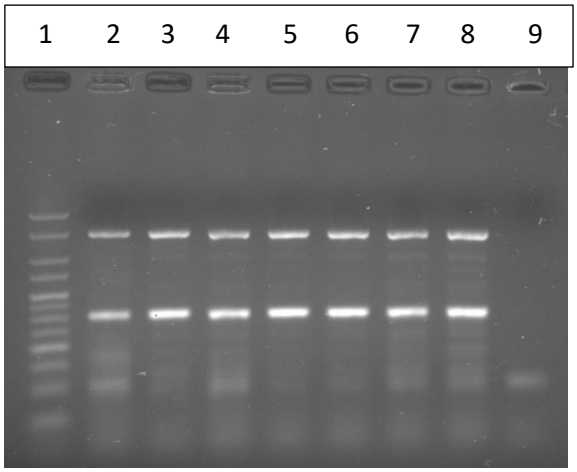


lincoln 2019-11-27_13h22m15s.tif

S.No	Strain Name	Multiplex genotyping				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	**PHYLOGROUP**
1	Hyper V ladder					
4	TL-28-W18	+	-	-	-	A
5	TL-36-W18	-	-	-	-	UNKNOWN
	A+C typing			**trpBA**	**trpAgpC**	
7	CW-23-W18			+	+	C
8	*E. coli* NCTC13352			+	+	C
11	MRD-45-S17			+	+	C
12	MRD-25-S18			+	+	C
13	CW-51-S17			+	+	C
	D+E TYPING			***trpBA***	***trpAgpE***	
2	PF-19-S18			+	+	E
3	CW-34-S18			+	+	E
6	PF-39-S17			+	+	E
9	PF-45-S17			+	-	Clade I
10	CW-18-S18			+	+	E
14	*E. coli* ATCC25922	-	+	+	+	B2
15	*E. coli* NCTC13352	+	-	+	-	A+C



lincoln 2019-10-08_14h43m16smultiplexforTLS17.tif

S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	*chu A*	*yja A*	*TspE4C2*	
1	HYPER V LADDER					
2	TL-87-A18	+	-	-	+	B1
3	TL-59-S17	+	-	-	+	B1
4	TL-76-S17	+	-	-	+	B1
5	TL-82-S17	+	-	-	+	B1
6	TL-69-S17	+	-	-	+	B1
7	TL-87-A18	+	-	-	+	B1
8	TL-75-S17	+	-	-	+	B1
9	DW	-	-	-	-	
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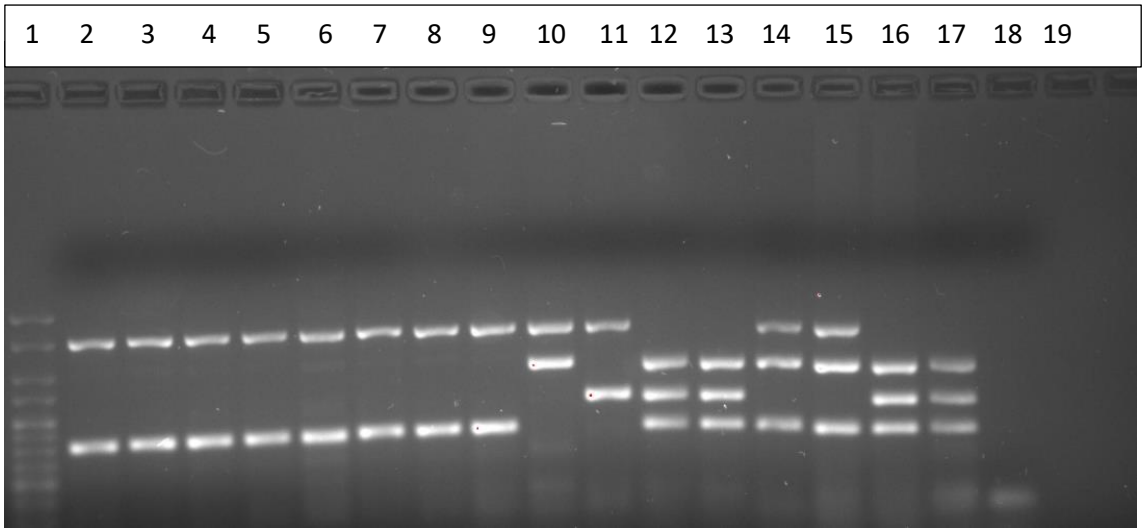
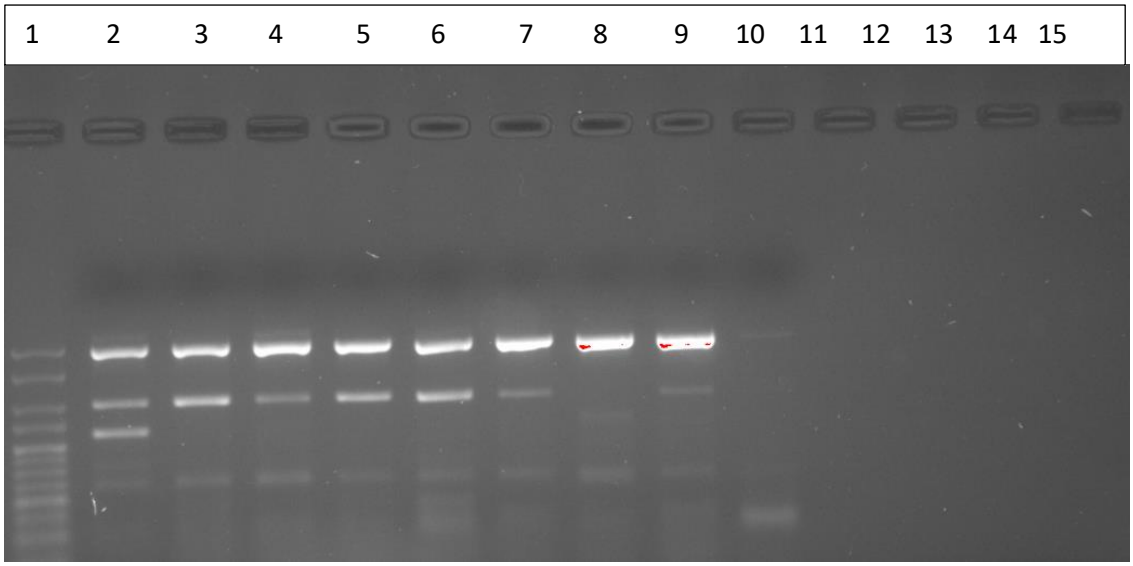


lincoln 2019-10-09_15h40m54squadruplex.tif

S.No	Strain Name	Quadruplex genotype				Phylogroup
		ArpA	***chu A***	***yja A***	***TspE4C2***	
1	HYPER V LADDER					
2	TL-60-S17	+	-	-	+	B1
3	TL-71-S17	+	-	-	+	B1
4	TL-72-S17	+	-	-	+	B1
5	TL-64-S17	+	-	-	+	B1
6	TL-58-S17	+	-	-	+	B1
7	TL-75-S17	+	-	-	+	B1
8	TL-61-S17	+	-	-	+	B1
9	TL-63-S17	+	-	-	+	B1
10	46	+	+	-	-	D+E
11	10	+	-	+	-	A+C
12	4	-	+	+	+	B2
13	A7	-	+	+	+	B2
14	A5	+	+	-	+	D+E
15	A2	+	+	-	+	D+E
16	B10	-	+	+	+	B2
17	*E. coli* ATCC25922	-	+	+	+	B2
18	DW					
19						
20						



lincoln 2019-10-09_15h47m51sD+E.tif

S.No	Strain Name	Phylo E genotyping				Phylogroup
				trpBA	***trpAgpE***	
1	Hyper V ladder					
2	CW-35-A18			+	+	E
3	CW-27-A18			+	+	E
4	CW-3-S17			+	+	E
5	CW-20-S17			+	+	E
6	MRD-55-A18			+	+	E
7	MRD-37-A18			+	+	E
8	MRD-49-A18			+	-	Clade I
9	MRD-26-A18			+	+	E
10	DW					
11						
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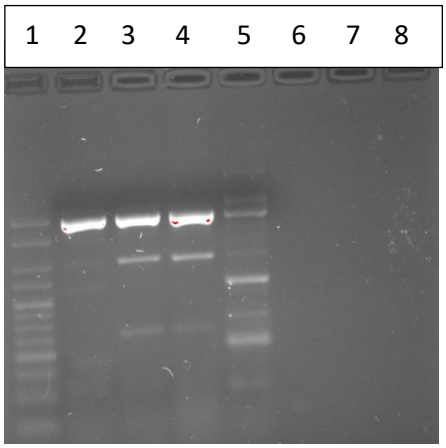

lincoln 2019-10-10_12h03m14sD+Ej and AC of otago esbl ctrls.tif

S.No	Strain Name	Phylo D+E genotyping				Phylogroup
				trpBA	***trpAgpE***	
1	Hyper V ladder					
2	46			+	-	D
3	A5			+	+	E
4	A2			+	+	E
5	**Phylo A+C genotyping**					
6	B10			+	+	C
7						
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