Phenotypic and Genotypic Characterisation of *Escherichia coli* O26 Strains Isolated from Cattle and Clinical sources in Australia

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Abstract

Escherichia coli (*E. coli*) O26 is the second most common serogroup of enterohaemorrhagic *E. coli* (EHEC) implicated in foodborne illness globally with outbreaks occasionally linked to the consumption of cattle related food products. This study was performed to gain insights into the phenotypic and genotypic characteristics of *E. coli* O26 circulating in Australian cattle and those strains from clinical cases. Initial characterisation determined that *E. coli* O26 are a genetically diverse group that can belong to a range of pathotypes including: EHEC, atypical enteropathogenic *E. coli* (aEPEC), potential EHEC (pEHEC) and non-toxigenic *E. coli* (NTEC) and split into two main clusters by pulsed field gel electrophoresis. When the preliminary characterisation data were combined with spatial and temporal data a subset of 40 out of 88 isolates comprising ten human and 30 cattle *E. coli* O26 isolates were selected for subsequent analysis.

The response of *E. coli* O26 strains in planktonic or biofilm forms to a range of antimicrobials and sanitisers and the possible influence of pathotype or strain source was assessed. The survival of *E. coli* O26 to disinfectants in a planktonic state varies depending on which stressor is applied. Disinfectants containing quaternary ammonium compounds or peracetic acid inhibited the growth of *E. coli* O26 whereas hypochlorite and acid anionic based disinfectants were not effective. It was also demonstrated that O26 strains of all pathotypes examined here had elevated MICs (1,024 to 4,096 µg/ml) to acetic, propionic, lactic and citric acids. *E. coli* O26 in planktonic state did not demonstrate variability in resistance when source or pathotype was considered.

To evaluate the survival capability of strains in biofilm state, biofilm formation on different surfaces was investigated. EHEC strains were more likely to form biofilms

after 48 hr than aEPEC strains on polystyrene plates. However, biofilm formation was generally enhanced with increasing incubation time and was further enhanced for all pathotypes when stainless steel and glass slides were used as a contact surface. Interestingly, investigating pellicle formation at the air-liquid interface indicated that pellicle formation was primarily produced by EHEC strains and NTEC while it was absent from aEPEC. The study also demonstrated that prophage insertion in *mlrA* (*yehV*), mutations in RpoS, lack of motility and failure to produce cellulose and/or curli was associated with reduced biofilm forming capacity. It was also demonstrated that biofilms provided a protective effect to *E. coli* O26 strains against the three sanitisers, previously shown to successfully control the growth of their planktonic counterparts, regardless of pathotype, source and the amount of biofilm formed.

The carriage of *stx* is a key factor in the ability of *E. coli* O26 to cause human disease. Stx bacteriophage insertion sites and the ability for *stx* to be gained or lost were investigated. It was found that Stx phages integrated into *wrbA*, *yehV* or near the *torS* gene, a novel not previously described insertion site. Although these loci were shown to be intact in *stx*-negative strains, this study was unable to detail the ability or frequency at which Stx phage are acquired by *stx*-negative O26 strains. The carriage of non Stx phage integrases was also assessed with pathotype specificity observed which likely promotes the differing genetic profiles observed between pathotypes. Comparative analysis of Stx phage induction, toxin production and *stx* expression (copy number) identified that lysogens were found to produce greater quantities of Stx than their parent counterparts. Overall, *stx* expression and Stx production were comparable between EHEC regardless of source thereby confirming the pathogenic potential of EHEC cattle strains.

Finally, the use of whole genome sequencing (WGS) provides evidence of the continuous evolution and plasticity of the genome of this organism and its ability to undergo genetic rearrangements and further confirmed the relationship between human and cattle strains. This study determined that while pathotypes of *E. coli* O26 appear to be highly clonal regardless of source, certain pEHEC and aEPEC strains share similar genetic profiles to EHEC strains and could rapidly transition to EHEC via the acquisition of *stx*.

E. coli O26 remains an organism of significance to global public health and cattle processing systems. This study has confirmed that cattle are a potential reservoir of EHEC strains capable of causing human disease and has demonstrated the ability of EHEC strains to survive in the presence of anti-bactericidal substances. Continued implementation of regular and effective sanitisation will assist in controlling the hazard and associated risk caused by this global public health and economically important pathogen to the food industry.

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Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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28/07/2017

Statement of contribution by others

Dr. Robert Barlow and Dr. Jeremy Brownlie assisted in conceiving and designing the experiments. Dr. Robert Barlow gave guidance on interpretation of results and provided advice on the draft versions of the thesis and the preparation of the manuscripts published and submitted as a part of this thesis. Salma Lajhar performed the experiments, acquired the data, interpreted the data and drafted the manuscript. All the data and figures used in this thesis and manuscripts were prepared by the candidate; except for aspects of bioinformatic analysis and Figures of pangenome PCA, the SNP PCA and methods 3.3, 3.6, 3.7, 3.8 (Chapter 5) which were prepared by Dr Theo Allnutt. Consent by all authors has been given for the inclusion of the publications in this thesis.

Dr. Robert Barlow Dr. Jeremy Brownlie

Publications and conference presentations relevant to the thesis.

Publications

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Conference Presentations

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Abbreviations

A/E	Attaching and effacing
adiA	Acid inducible arginine decarboxylase
adiC	Acid inducible arginine decarboxylase antiporter
aEPEC	Atypical enteropathogenic *E. coli*
AFI	Acid fitness island
ATCC	American type culture collection
Bfp	Bundle forming pilus encoding gene
CadB	lysine–cadaverine antiporter
cAMP	Cyclic adenosine monophosphate
CDC	Centres for disease control and prevention
CDS	Coding sequences
CFU	Colony-forming unit
CGE	Centre for genomic epidemiology
CIP	Ciprofloxacin
CRP	cAMP receptor protein
CSFAs	Short-chain fatty acids
DAEC	Diffuse-adhering *E. coli*
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
eae	*E. coli* attachment and effacing gene
EAEC	Enteroaggregative *E. coli*
EAHEC	Enteroaggregative haemorrhagic *E. coli*
Ecf	*eae* positive conserved fragments
EEA	European economic area
EHEC	Enterohaemorrhagic *E. coli*
ehxA	Enterohaemolysin A

EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
Escs	*E. coli* secretions
Esp	*E. coli* secreted protein
EspP	*E. coli* serine protease
ETEC	Enterotoxigenic *E. coli*
Gad	Glutamate decarboxylase
GadC	Glutamate decarboxylase antiporter
Gb3	Globotriaosylceramide
H	Heat labile flagella antigen
H^-	Non-motile
HC	Haemolytic colitis
HdeA	Histone-like nucleoid dependent expression A
Hly	Haemolysin
H-NS	Histone-like nucleoid-structuring protein
HS	High sensitivity
Hsp31	Heat shock protein31
HUS	Haemolytic uremic-syndrome
Iha	Iron-regulated gene A (IrgA) homologue adhesin
IL	Interleukin
IVOC	*In vitro* organ culture
katP	Catalase-peroxidase
kDa	Kilo Dalton
LB	Luria-Bertani
LdcI	Lysine decarboxylase
LEE	Locus of enterocyte effacement
Ler	LEE regulator
LPS	Lipopolysaccharide

MAP	Mitochondrion-associated protein
mDa	Megadaltons
MLST	Multi locus sequence typing
MLVA	Multi-locus variable number tandem repeat analysis
MUSCLE	Multiple sequence comparison by log-expectation
NCTC	National Collection of Type Cultures
NF-kB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
Nle	Non LEE effector
NM	Non motile
NTEC	Non-toxigenic *E. coli*
O-PS	O-polysaccharide
ORF	Open reading frame
PAIs	Pathogenicity islands
PCA	Principal components analysis
PCR	Polymerase chain reaction
pEAF	Plasmid-EPEC adherence factor
pEHEC	Potential enterohaemorrhagic *E. coli*
PFGE	Pulsed-field gel electrophoresis
PotE	Ornithine/putrescine antiporter
QAC	Quaternary ammonium compounds
RpoE	Heat shock sigma factor
RpoS	Stationary phase σS factor
RTX	Repeats in toxin
Sep	Secretion of *E. coli* proteins
SNPs	Single nucleotide polymorphisms
SpeF	Ornithine decarboxylase
STEC	Shiga toxin producing *E. coli*
Stx	Shiga toxin

TccP	Tir-cytoskeleton coupling protein
tEPEC	Typical enteropathogenic *E. coli*
TIM	TNO (gastro-) Intestinal Model
Tir	Translocated intimin receptor
TNFα	Tumour necrosis factor alpha
ToxB	Potential adhesin
TTSS	Type-III secretion system
USA	United states of America
VTEC	Vero-toxin-producing *E. coli*

Chapter 1

Literature Review

1.1 **Introduction**

Escherichia coli (*E. coli*) is the most common gram negative facultative anaerobic normal intestinal flora that colonises the intestinal mucous layer of the colon and coexists with its host in the gastrointestinal tract in a mutually beneficial relationship (Rasko et al., 2008, Tchaptchet and Hansen, 2011). However, some *E. coli* strains can be pathogenic and are among the most important etiological agents of human diarrhoeal disease and extraintestinal diseases (Rasko et al., 2008). *E. coli* can be split into pathotypes based on the virulence profiles. Some of the important pathotypes include: enterohaemorrhagic *E. coli* (EHEC) which is a subset of Shiga toxin producing *E. coli* (STEC) or Verotoxin-producing *E. coli* (VTEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC) and diffuse-adhering *E. coli* (DAEC) enteroaggregative *E. coli* (EAEC) [Reviewed in (Nataro and Kaper, 1998)] and enteroaggregative haemorrhagic *E. coli* (EAHEC) (Bielaszewska et al., 2011).

Although different *E. coli* pathotypes have been associated with human disease, EHEC cause foodborne disease and constitute the most common group firmly associated with bloody diarrhoea that can lead to life threatening sequelae such as haemolytic uremic-syndrome (HUS; a triad of non-immune microangiopathic haemolytic anaemia thrombocytopenia and nephropathy) and haemolytic colitis (HC) (Paton and Paton, 1998, Delannoy et al., 2013b). EHEC strains typically harbour both virulence determinants Shiga toxin (*stx*) and attaching and effacing gene (*eae*). Contact with animals on farms, consumption of contaminated meat, beef sausage, raw milk and raw milk products, water, spinach, sliced watermelon, clover sprouts, blueberries and strawberries have all been described as ways in which humans can be exposed to EHEC (Hiruta et al., 2001, Ethelberg et al., 2009, Hoshina et al., 2001, Lynch et al., 2012). Epidemiological surveillance indicates that *E. coli* O157:H7 and six non-O157

serogroups known as the big 6 EHEC such as O26, O45, O103, O111, O121, O145 are the most frequently encountered serogroups implicated in sporadic and outbreak cases (EFSA and ECDC, 2016a, Scallan et al., 2011, Vally et al., 2012). As cattle are the primary reservoir of epidemiologically significant EHEC (EFSA and ECDC, 2016a, Ethelberg et al., 2004, Ethelberg et al., 2009, Scallan et al., 2011), strains of O157:H7 and six non-O157 serogroups in the mid-1990s (USDA and FSIS, 1999) and in 2012 respectively (USDA and FSIS, 2012) were declared as adulterants in raw ground beef and in raw, non-intact beef.

After O157, *E. coli* O26 is the second most common serogroup associated with EHEC illness. *E. coli* O26 have been isolated from beef and dairy cattle and have been implicated in several foodborne outbreaks linked to the consumption of beef products (EFSA and ECDC, 2016a, Ethelberg et al., 2009). The Australian cattle industry is a major producer and exporter of beef and beef products therefore investigating and controlling these pathogens is of importance to the export meat trade to maintain and expand access to markets that regulate for the presence of EHEC. In comparison to O157, limited information exists regarding the phenotypic and genotypic characteristics of *E. coli* O26 from Australia. For instance, little is known about the survival and growth of *E. coli* O26 of various pathotypes when exposed to stress conditions and whether survival is based on the cells being in a planktonic or biofilm state. Similarly little is known about the pangenome and the phylogeny of Australian O26 strains.

1.2 Rationale for studying O26 serogroup, industry and regulations

Australia is one of the top three beef exporting countries in the world, exporting 74% of its total beef and veal production to 84 countries in 2016 (Figure 1.1). The export of red meat

and livestock are a significant contributor to the nation's economy with the red meat industry employing 200,000 people including on-farm production, processing and retail (MLA, 2016). The export meat trade value was $8.5 billion in 2015-2016 and the live cattle exports were valued at $1.5 billion in 2015-2016 (MLA, 2016). Contamination of food with EHEC can negatively impact industry and lead to product recall, economic loss and of businesses (Ollinger and Mueller, 2003). Contamination of red meat products and fresh produce with EHEC can occur during slaughter or through the use of water contaminated with cattle faeces in horticulture. It has been reported that EHEC serogroups detected in bovine meat are the most common serogroups causing human infection with EHEC O26 being the most detected non-O157 serogroup in food (bovine meat, ovine meat, milk and dairy products) accounting for 25.6% of any non-O157 EHEC-associated disease (EFSA and ECDC, 2016a). In the USA in 2016, the volume of recalled bovine meat was 591,869 pounds (~268,467Kg) with over 241180 pounds (~109,397Kg) recalled due to contamination of bovine meat with the top-seven EHEC (FSIS, 2016). In Australia, the main EHEC of clinical significance found to be shed in cattle faeces are O157, O26 and O111. Although they were more likely to shed EHEC at low concentration (less than 3 $\log_{10}$ MPN g^{-1} faeces), cattle with EHEC counts greater than 3 $\log_{10}$ MPN g^{-1} faeces was higher for EHEC O26 (33%) and O157 (29%) than that of O111 (Mellor et al., 2016).

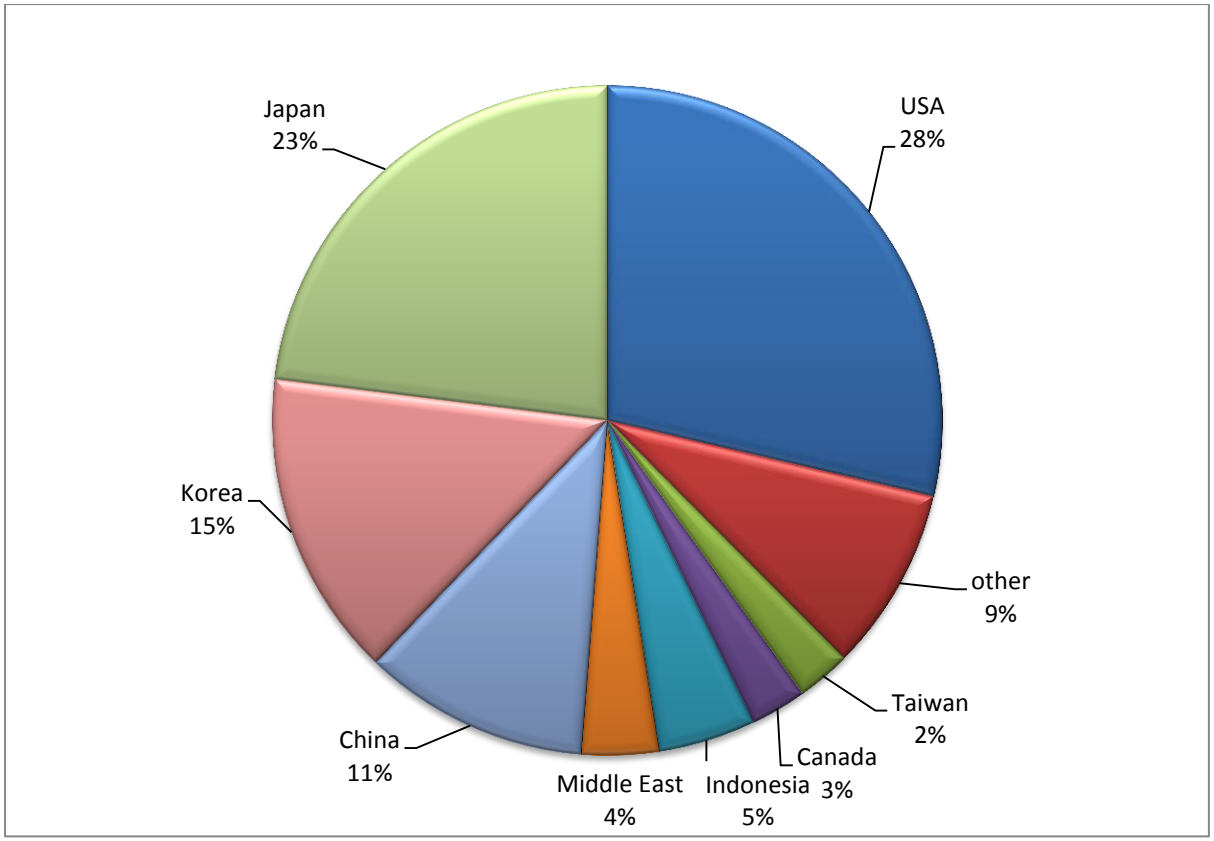

Figure 1-1. Australian beef and veal exports from 2015-2016. Australian beef industry exports a large amount of its product to the USA and Japan.(MLA, 2016)

E. coli O26 as a member of big 6 EHEC is of importance to the Australian export meat trade which has implement testing measures to maintain export market access and to deliver safe and hygienic products for consumers. The United States of America (USA), which declared six non-O157 serogroups including O26 as adulterants in 2012 is the biggest market for Australia. Furthermore, EHEC O26 is the most common non-O157 serogroup implicated in serious foodborne outbreaks in the USA, accounting for 32% of non-O157 associated disease in 2015 (CDC, 2015b). A key aspect to ensure safe and hygienic processing and maintain access to markets that regulate for EHEC is to implement food safety and microbiological risk management that is based on the epidemiology of a pathogen. Therefore, characterising strains isolated from a source that are perceived to be implicated in human infections and

comparing them to clinical isolates in order to provide meaningful information on the key characteristics of this clinically and economically important pathogen to the Australian red meat exporters is warranted.

1.3 *E. coli* O26 epidemiology

EHEC O26 has been identified as a major contributor to sporadic and outbreak cases of diarrhoea and post-diarrhoeal HUS worldwide. In Australia, the average EHEC notification per 100,000 population over 11 years period (from 2000-2010) was 0.4 (Vally et al., 2012) and increased to 0.6 in 2016 (OzFoodNet, 2016, OzFoodNet, 2017). The proportion of EHEC O157 and non-O157 isolates is shown in Figure 1.2 with the most notified serogroups being O157; O111 and O26 (OzFoodNet, 2015, Vally et al., 2012).

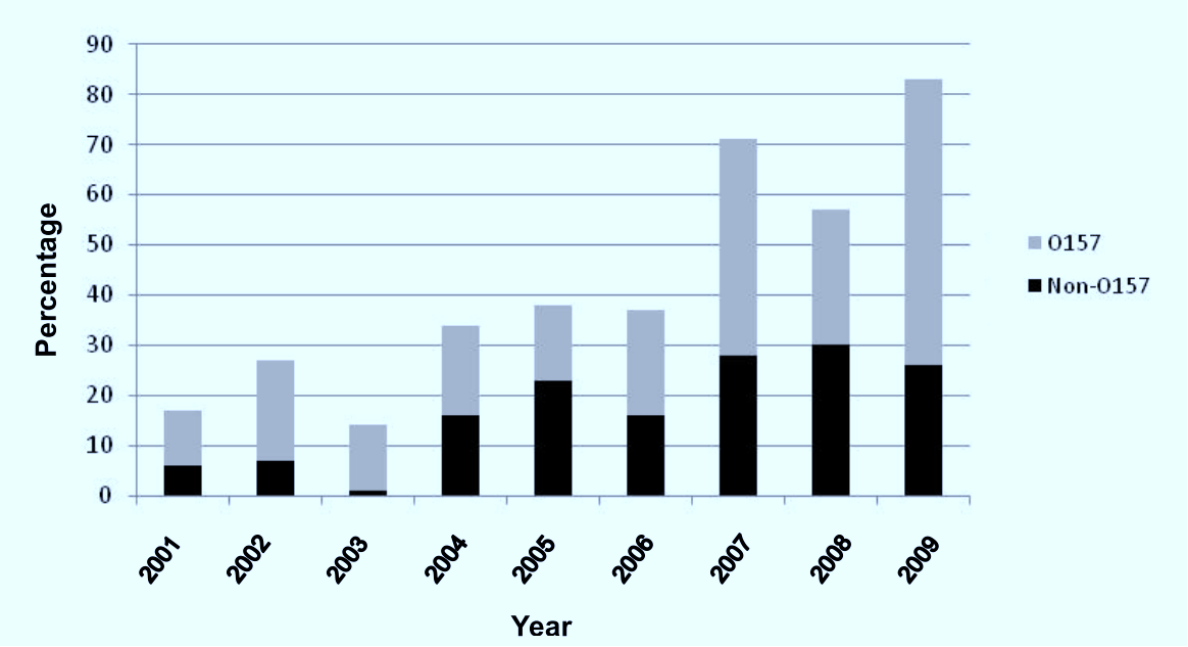

Figure 1-2. Proportion of EHEC O157 and non-O157 isolates (of those with a known serogroup) from 2001-2009. Reproduced with permission from the publisher (Vally et al., 2012).

Since 2010, there has been an increase in the reporting of outbreaks caused by EHEC O26 in Europe/ European Economic Area (EEA) countries (Austria, Belgium, the Czech Republic, Denmark, France, Germany, Hungary, Ireland, Italy, Lithuania, Luxembourg, the Netherlands, Norway, Poland, Romania, Slovenia, Spain, Sweden and the United Kingdom). The incidence of infection was high particularly in Ireland (32%) followed by Germany (15%) and Sweden (12%) (EFSA and ECDC, 2016b). Overall, the most reported non-O157 serogroup in Europe in 2015 was O26 accounting for 14% of total reported cases (3624) which was higher than that reported in 2014 (12.2% of 3654) and 2013 (12.7% of 3737) (EFSA and ECDC, 2016a). Community and HUS associated outbreaks have been reported in a number of European Union (EU) member states including Italy, Germany, Austria, Denmark and France (Allerberger et al., 2003, Espié et al., 2006, Ethelberg et al., 2004, Ethelberg et al., 2009, Gerber et al., 2002, Sayers et al., 2006, Tozzi et al., 2003). In 2015, of 2,350 hospitalised cases, HUS due to O26 accounted for 27.9% which was equal to that reported for O157 (EFSA and ECDC, 2016a). In the United States (USA), EHEC non-O157 serogroups caused approximately 168,698 cases of illness between 2000 and 2008 including 271 hospitalisations and EHEC O26 was the predominant non-O157 EHEC responsible for human disease (Scallan et al., 2011). Additionally, the Centres for Disease Control and Prevention (CDC) reported that *E. coli* O26 is the second most causative agent of foodborne disease outbreaks, outbreak-associated illnesses, and hospitalisation among STEC in 2014 and 2015 with three single outbreaks in 2014 and four outbreaks in 2015 (CDC, 2014, CDC, 2015b). Three of the four outbreaks were multistate (CDC, 2015b). *E. coli* O26 serogroup was also one of the most frequent non-O157 isolated from children with bloody diarrhoea and HUS in Brazil (Vaz et al., 2006). In Japan, *E. coli* O26 was the most common cause of childcare facility-related outbreaks from 2010 to 2013, accounting for 39% of EHEC strains isolated among 66 reported outbreaks (Kanayama et al., 2015).

1.4 *E. coli* O26 prevalence among cattle

The apparent increase in the number of clinical cases attributed to EHEC O26 serogroup combined with the prevalence of pathogenic strains of *E. coli* O26 in cattle and its raw products has resulted in a number of surveillance studies to estimate the prevalence of this pathogen in cattle. In an Irish study, the average proportion of EHEC O26 based on the investigation of bovine rectal faecal swabs in two seasonal cycles was 6-7%, and the prevalence of virulence determinants of strains was 36% (*stx*, *eae* and *ehx*) (Lynch et al., 2012). In a Canadian study, *stx* genes were present in combination with *eae* and *ehx* genes in 93% of *E. coli* O26 strains (Geue et al., 2009). In Scotland, farm-level and faecal pat–level prevalences were estimated for *E. coli* O26 obtained from 338 farms and 6,086 faecal pat samples with only 12 farms and 24 faecal pats samples testing positive for EHEC O26 (Chase-Topping et al., 2012). In an Australian study, the percentage of *E. coli* O26: H11 (1.7%) detected in dairy cattle faeces was nearly equal to that of EHEC O157 (1.9 %) (Cobbold and Desmarchelier, 2000). This finding agrees with another recent study which demonstrated that the prevalence of *E. coli* O26 in Australian beef cattle faecal samples was very low (1%) (Mellor et al., 2016). Although the occurrence of EHE CO26 in the above mentioned studies was relatively low, the prevalence of *stx*-positive strains in cattle suggests that they represent a potential risk to human health as the uptake of *stx*-encoding bacteriophages could confer *E. coli* O26 with greater pathogenic potential for humans.

1.5 *E. coli* O26 pathotypes

According to their virulence markers, *E. coli* O26 serogroup can be subdivided into EHEC and EPEC. Both EHEC and EPEC are characterised primarily by the presence of intimin encoded by *eae* and the ability to form attaching and effacing (A/E) lesions on intestinal cells

(Nguyen et al., 2006) (Figure 1.3). However, in addition to *eae*, EHEC strains carry *stx*. EPEC can be split into two sub-pathotypes with strains carrying EPEC adherence factor (pEAF) plasmid encoding bundle forming pili *bfp* termed typical EPEC (tEPEC) (Piazza et al., 2013) while atypical EPEC (aEPEC) are strains that lack the EAF plasmid and hence do not produce Bfp (Bugarel et al., 2011a). Strains of other pathotype of *E. coli* O26 have been described in the literature. These strains lack *stx* but carry *eae* and other EHEC virulence determinants such as *espP* and *ehx* and are termed potential EHEC (pEHEC). This pathotype has been also referred to as EHEC-like, EHEC derivatives or aEPEC closely related to EHEC (Bugarel et al., 2011a, Leomil et al., 2005). pEHEC strains have been isolated from clinical samples (Bielaszewska et al., 2006, Karch et al., 1992) and food samples (ground beef, salad, milk and milk filters) that also contain EHEC strains (Imamovic and Muniesa, 2011, Trevisani et al., 2014)

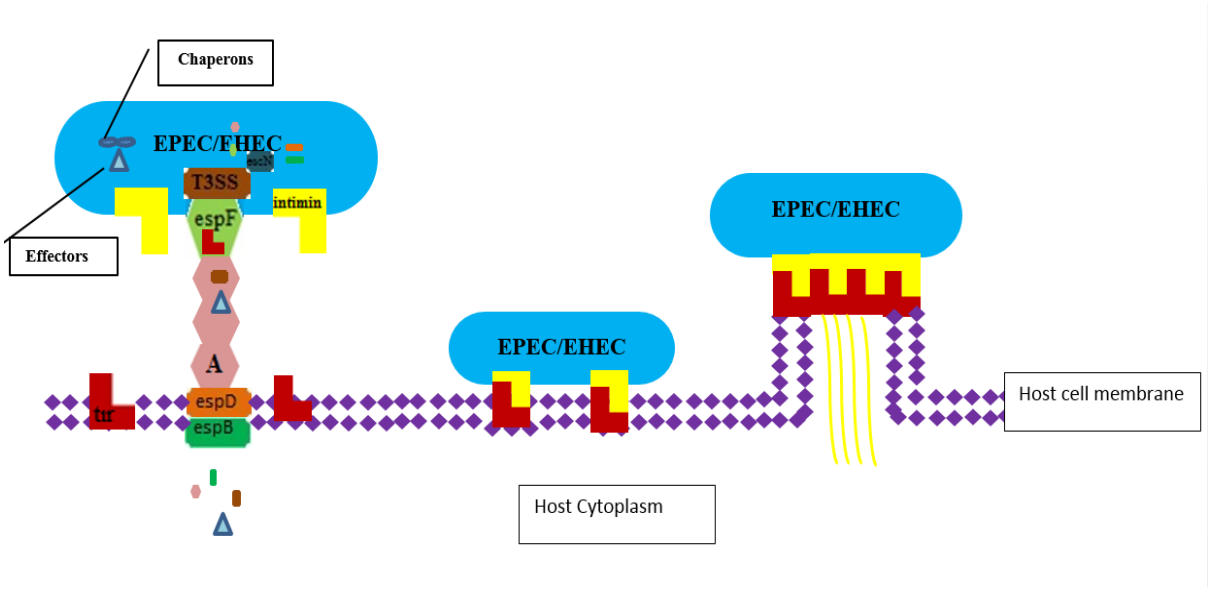

Figure 1-3. Attaching and effacing (A/E) lesions on intestinal cells. Bacteria are linked to its host by T3SS, EspF, EspB, EspA and EspD; intimin interact with its translocated receptor Tir, other T3SS proteins and effectors will transfer through the assembled T3SS. The A/E lesion is characterised by intimate attachment to epithelial cells and destruction of intestinal microvilli and forming actin pedestals' structure beneath the attached bacteria. Adapted from (Nguyen and Sperandio, 2012).

1.6 Survival and persistence of *E. coli* O26

The survival and persistence of *E. coli* O26 in food processing environments represents a significant risk factor for human infection and threat of potential revenue loss if contaminated food product were recalled or held. The risk to humans will largely depend on the survival and adaptation capabilities of the pathogen in environments they encounter. The conditions for the survival of *E. coli* O26 once excreted from the cattle gut are generally less favourable as the organism will encounter fluctuating levels of energy sources, oxygen, temperatures, disinfectants, low pH and osmolarity (reviewed in (Nguyen and Sperandio, 2012)). However, *E. coli* O26 that are able to survive for extended periods of time by adaptation to such conditions can cause cross contamination of meat and produce on farm and can be further transmitted to uncontaminated produces during food processing and packaging (Cobbold and Desmarchelier, 2000, Fukushima et al., 1999, Espié et al., 2006). In comparison to *E. coli* O157, limited studies have been conducted to investigate stress response of *E. coli* O26.

1.6.1 Survival in acidic environments

The capability of some strains of EHEC to survive different acidic environments encountered in food, stomach and intestine is an important factor that determines its ability to cause gastrointestinal illness (Arnold et al., 2001, King et al., 2010). Adaptation to acid stress encountered during passage through the low pH medium in the stomach (between 1.5-3) and the relatively neutral pH of the intestine, which contains high concentrations of short-chain fatty acids (CSFAs) such as lactic acid, acetic acid, and propionic acid, is an essential factor in the colonisation and survival of enteric pathogens (Arnold et al., 2001). The number of foodborne outbreaks involving acidic food caused by EHEC and the detection of EHEC O26

in acidified and fermented food has drawn the attention to the acid resistance properties of this pathogen.

E. coli utilise a number of acid resistance mechanisms to prevent the effect of acidic stresses including enzyme-based acid stress response systems and the chaperone-based stress response (Table 1.1). Most genes involved in the acid resistance mechanisms are located on a 15 kb genomic island known as the acid fitness island (AFI) on the *E. coli* chromosome (Tramonti et al., 2008). The AFI is repressed by histone-like nucleoid-structuring protein (H-NS) and controlled by the stationary phase σ^S factor (RpoS) (Carter et al., 2012). Furthermore, the island is conserved in *E. coli* O157:H7 and EHEC non-O157 strains (O26:H11, O103:H2, and O111:H); although in *E. coli* O157:H7 the AFI is much larger (Carter et al., 2012).

Table 1-1. Summary of acid stress response mechanisms

Acid stress response	Induction requirements and function	Major proteins components	Reference
Oxidative system (AR1)	• Stationary phase alternative sigma factor • Cyclic AMP (cAMP) receptor protein (CRP) and • Aerobic growth condition	unknown	(Castanie-Cornet et al., 1999)
Glutamate decarboxylase system (GDAR-AR2)	• Extracellular glutamate • Stationary phase or Log phase • Cells exposed to SCFAs • Anaerobic and acidic condition • Highest protection against extreme acidic condition (pH 2)	• Glutamate Decarboxylase (GadA & GadB) • Antiporter (GadC)	(Castanie-Cornet et al., 1999)
Arginine decarboxylase system (ADAR-AR3)	• Extracellular Arginine • Cells exposed to SCFAs • Anaerobic and acidic environment • High Protection against extreme acidic condition	• Acid inducible arginine decarboxylase (AdiA) • Antiporter (AdiC)	Castanie-Cornet et al., 1999)
Lysine decarboxylase system (LDAR-AR4)	• Anaerobic • Protection at mild pH ~4.4 • Excess lysine and phosphate starvation	• Lysine decarboxylase LdcI (CadA) • Lysine cadaverine antiporter (CadB)	(Moreau, 2007)
Ornithine decarboxylase system (ODAR-AR5)	• Extracellular ornithine • Cell protection at pH 5.2	• Ornithine decarboxylase (SpeF) • Ornithine /putrescine antiporter (PotE)	(Kashiwagi et al., 1991)
Chaperone proteins	• Stationary phase • Cell protection at Low pH (2)	• HdeA and HdeB and Hsp31 (*hchA*)	(Carter et al., 2012)

Bergholz and Whittam (2007) studied the effect of gastric acid (pH 2.5) on the survival of stationary phase acid adapted (pH 3.5 or 7.0) EHEC O157:H7, O26:H11 and O111:H8. Overall, the survival of cells stored at 4^{o}C in pH 3.5 prior to exposure to gastric acid in a stomach model system was better than the survival of cells stored at 22^{o}C at the same pH before treatment with gastric acid which suggest that storage at 4^{o}C resulted in production of cold shock proteins which led to cross protection under the extreme acidic environment (Smith and Fratamico, 2012). Studies on the effect of food acidity on the survival of EHEC

E. coli O26 demonstrated that there is a great variability in survival of EHEC *E. coli* O26 when inoculated into acidic food (Molina et al., 2005), acidified broth (Molina et al., 2003) and fermented products (Duniere et al., 2011). Beier et al. (2016) found that EHEC non-O157 including O26 had high MICs (1024 to 4096 µg/ml) for acetic, lactic, citric and propionic acids. Overall, the literature suggests that survival of *E. coli* is serotype, strain and food matrix dependent and variation in the survival data could be attributed to cross-protection, expression of virulence factors under stress and resistance system activation on exposure to stress (Miszczycha et al., 2013) as well as differing methods utilised to measure the survival capability.

1.6.2 Resistance to biocides

E. coli O26 may persist in food processing environments posing a serious risk to public health and potentially resulting in economic losses (Todd, 2004). Detection of *E. coli* O26 isolates in various food environments, food equipment and surface material and identifying them as a causative agent for a number of foodborne outbreaks creates the need for identifying critical control points during production and processing to implement prevention strategies to control this pathogen. Different biocides are used in the form of antimicrobial agents such as sanitisers and disinfectants. The microbicidal activity of sanitisers and disinfectants is mostly based on quaternary ammonium compounds (QAC), organic acids, chlorine and peracetic acid. However, bacteria can withstand lethal levels of biocides by using a number of strategies such as alterations to the cell wall permeability, reduction in the influx of chemicals into the bacterium to reduce their accessibility and acquisition of new genetic materials encoding resistance to disinfectants from other organisms (Beier et al., 2013).

Regular evaluation of the effective spectrum of disinfectants is required to ensure that proper sanitisation is achieved and to prevent the spread of pathogens. Resistance of *E. coli* recovered from various environments to a variety of biocides has been reported. Beier et al. (2016) investigated the disinfectant profile for non-O157 including O26 human and cattle isolates and found that 4.4 % of *E. coli* O26 isolates were resistant to chlorhexidine whereas resistance to QAC compounds was reported at 77.7% for Food Service Sanitiser (FSS), 75.5% for F-25 Sanitiser (FS25) and resistant to Final Step 512 Sanitiser (FS512) was observed at 64.4%. Variations between the response of different strains of *E. coli* to a range of disinfectants was demonstrated by Škaloud et al. (2003). This data suggests that the response of different of *E. coli* O26 isolates may also be highly variable.

1.6.3 Antimicrobial resistance (AMR)

Resistance to antimicrobials by pathogenic bacteria is a significant risk to human and animal health and might limit the future usefulness of these compounds in human and veterinary medicine particularly antimicrobial agents of critical importance to human health including cephalosporins (3^{rd}, 4^{th} and 5^{th} generation), glycopeptides, macrolides, ketolides, polymyxins and quinolones. To survive in the presence of an antimicrobial agent, bacteria can prevent the antimicrobial agent from penetrating into the cell wall, modify or degrade antimicrobial agents or pump the antimicrobials out of the cell by efflux pumps (Beier et al., 2013). Although treatment of EHEC infection with antimicrobials remains controversial as it may increase risk of developing HUS, antimicrobials may be used in clinical practice for treatment of severe EHEC infection.

An increase in the emergence and spread of antimicrobial resistance and multidrug-resistant *E. coli* have been reported in medical and veterinary surveillance data (Petty et al., 2014,

Ramos et al., 2013, Tadesse et al., 2012). A retrospective study of 1729 *E. coli* isolates recovered from human and food animal samples during 1950–2002 revealed an increased resistance to ampicillin, sulphonamide and tetracycline in human isolates and an upward trend in resistance of animal isolates to ampicillin, sulfonamide and gentamicin (Tadesse et al., 2012). In addition, 63.6% of isolates collected post 2000 were multidrug resistance (≈3 antimicrobial classes) compared to 7.2% during the 1950s. Multidrug resistant EHEC O26:H11 strains were responsible for an outbreak of diarrhoea in a nursery in Japan (Hiruta et al., 2001). EHEC strains that caused the outbreak had multidrug resistance to ampicillin, tetracycline, kanamycin, streptomycin and sulfamethoxazole. In another study, 5.3% of *E. coli* O26 from food animals (n=19) and 15.4% of human isolates (n=26) displayed an AMR phenotype with sulfisoxazole-streptomycin-tetracycline the most common profile in animal isolates whereas sulfisoxazole-streptomycin was the most observed profile in clinical isolates (Beier et al., 2016). A genotypic investigation of AMR in *E. coli* O26 revealed that 70% (n=42) of the USA cattle isolates exhibited AMR with 13 isolates exhibiting multidrug resistance (Gonzalez-Escalona and Toro, 2016).

In Australia, antimicrobial agents are used in cattle production for the prevention and control of bacterial associated diseases. Currently, limited data are available about the prevalence of AMR in *E. coli* O26 isolates in Australia as the majority of studies assess the status of antimicrobial agents to *E. coli* in general and not to particular serotypes. Barlow et al. (2004) conducted a survey on the prevalence of AMR in food purchased at retail outlets in Australia and concluded that although the level of AMR in general was low, particular food/ bacterium combinations were identified that harboured higher levels of AMR. A recent study assessed the AMR status in Australian cattle populations at slaughter by testing faecally-derived isolates from beef cattle (n=469), dairy cattle (n=155), and veal calves (n=176). The study confirmed the same finding of low AMR among the tested animal groups with susceptibility

levels of *E. coli* isolates ranging from 92.1% to 96.8%. In addition, low resistance to antimicrobials of high importance in human medicine was also observed (Barlow et al., 2015). Although AMR has been reported as low in Australia, there is a need to monitor trends in AMR development in order to control the emergence of AMR pathogens.

1.6.4 Biofilms

One of the ways for *E. coli* O26 to persist in different environments, including food processing establishments, is through the formation of biofilms. Biofilms are microbial sessile communities that are embedded in a self-produced matrix of extracellular components and extracellular DNA (eDNA), providing the microbial community with protection against environmental stress (Nesse et al., 2014, Berne et al., 2015). Formation of biofilms in most *E. coli* strains of different serotypes is dependent on a long, thin, wiry surface fibre known as curli fimbriae and synthesis of cellulose (Wang et al., 2012, Chen et al., 2013). Biosynthesis of cellulose requires the bcsABZC operon whereas both csgDEFG and csgABC operons are responsible for curli biosynthesis (Romling et al., 1998, Bokranz et al., 2005). The expression of curli and cellulose is under the control of DNA-binding transcription factor CsgD (Romling et al., 1998, Bokranz et al., 2005). CsgD regulation requires protein transcription factors (GGDEF/EAL) allowing *csgD* expression to be triggered by a number of stimuli such as temperature, pH, oxygen concentration, osmolality, and various nutrients. Such a process includes high number of regulators that can cause differences in the expression of *csgD* between strains and/or serogroups through establishment of different regulatory pathways (Romling et al., 1998, Ogasawara et al., 2010). One of the regulatory factors is a MerR-like regulator (*mlrA*; renamed from *yehV*) which binds the *csgD* promoter to enhance biofilm formation through RpoS-dependent transcription of *csgD*. Recent studies demonstrated that insertion of a prophage such as Stx- encoding bacteriophage in the *mlrA* loci of O157:H7 and

the top six non-O157 serogroups can result in loss of *mlrA* function and limit biofilm formation (Chen et al., 2013, Uhlich et al., 2013).

E. coli can attach, colonise and form biofilms on various contact surfaces (Figure 1.4) including surfaces commonly used in food processing environments and at several temperatures (Nesse et al., 2014, Kumar and Anand, 1998). Such a process include two steps: initial weak interactions (reversible) between the bacteria and the substratum followed by direct contact (irreversible attachment) between the bacteria and the substratum which is facilitated by cell wall surface structures such as: lipopolysaccharide, pili (Fimbriae), flagella and adhesins (Srey et al., 2013, Goulter et al., 2009). Once the bacteria are irreversibly attached to the surface, their removal requires application of physical methods (scrubbing or scraping) or breaking down the attachment forces and the cell wall (enzyme, sanitisers) (Bridier et al., 2011, Srey et al., 2013, Goulter et al., 2009). Equivocal data is present in the literature regarding the concordance between the attachment to different surfaces (Nesse et al., 2014, Rivas et al., 2007, Park and Chen, 2015). The ability of *E. coli* strains of different serotypes to attach and form biofilms is influenced by a variety of factors including mode of growth, media composition, temperature, hydrophobicity, pH, eDNA formation, QS-based extracellular cell–cell-signalling systems, surface charge, cell wall structures, food surface properties and food and organic residues (Uhlich et al., 2014, Wang et al., 2012, Rivas et al., 2005, Hood and Zottola, 1997, Burmolle et al., 2006).

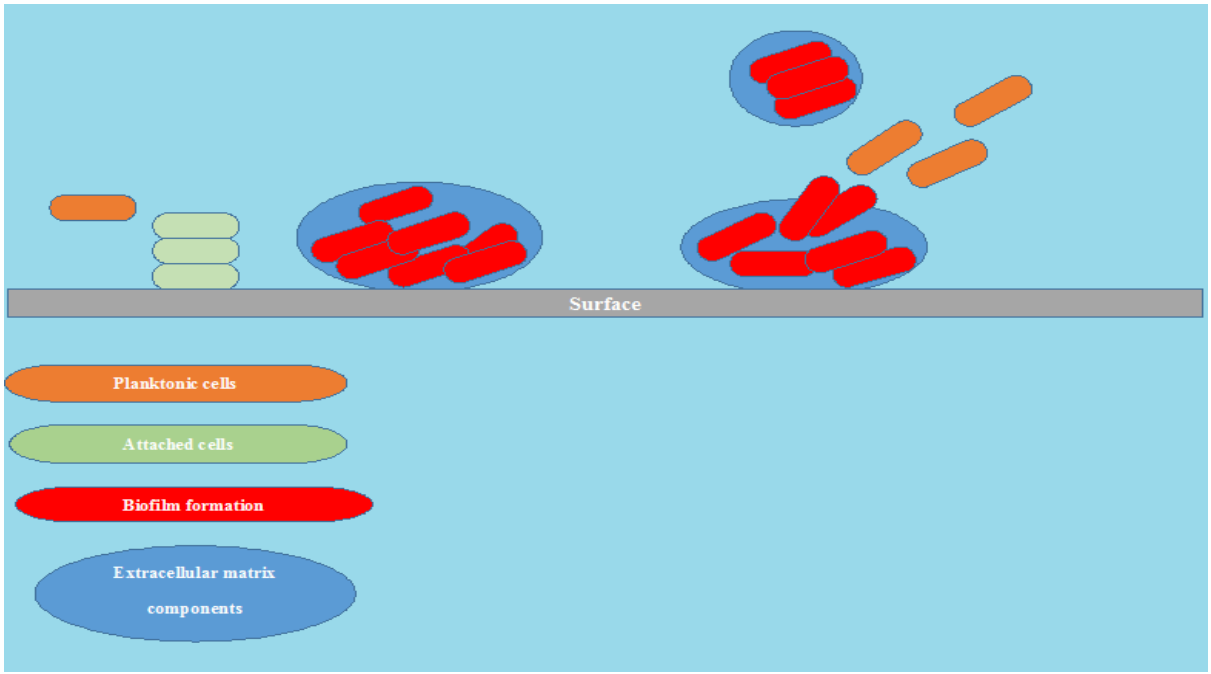

Figure 1-4. Steps of bacterial attachment and biofilm formation on surfaces. Planktonic cells attach to a surface (weak or reversible interaction) followed by irreversible attachment (direct contact between the bacteria and the surface) and finally production of extracellular matrix which provide the microbial community with protection against environmental stress. Cells within biofilm can disperse from the biofilm matrix as planktonic cell or biofilm.

The ability of strains to survive and persist in environments such as food processing facilities is complex. Once bacteria land on a substratum, bacterial cells could interact with other microorganisms and establish multispecies biofilm community (Hood and Zottola, 1997, Burmolle et al., 2006). For instance, resident microflora such as *Acinetobacter calcoaceticus* isolate was found to enhance biofilm formation by *E. coli* O157:H7 (Habimana et al., 2010). In addition, the influence of the substratum to which the bacteria would attach and the pre-exposure of substratum to food or meat residues for extended period of time could alter the strength of the attachment for a number of bacteria including *E. coli* by increasing or inhabiting the attachment process. For example, substratum soiled with whole milk reduced bacterial attachment and subsequently biofilm formation whereas pre-exposure of the substratum to beef juice extract offered protection for bacteria within the biofilm matrix from the surrounding stressors (Noyce et al., 2006, Helke et al., 1993)

Strains forming biofilms can be resistant to antibacterial intervention (including disinfectants) making their inactivation a crucial challenge in various environments (Wang et al., 2012, Uhlich et al., 2006). Some studies suggest that curli positive strains are much more tolerant to disinfectant treatment than curli negative EHEC O26 (Wang et al., 2012) while in other studies the same conclusion could not be made (Vogeleer et al., 2015). Differences between studies could be due to the panel of strains used, heterogeneity of the strains, sensitivity of the method and number of strains. There is also limited knowledge regarding the biofilm-forming capacity of *E. coli* O26 of different pathotypes from human and cattle. Overall, studies suggest that biofilm formation is important for surviving disinfectant treatment and that biofilms in food-producing facilities act as a source of bacteria that may contaminate food products causing human infections and severe illness (Nesse et al., 2014, Wang et al., 2012). Therefore, investigating factors that influence biofilm formation and attachment will contribute to controlling biofilms and thereby human illness.

1.6.5 Role of virulence factors in survival and persistence

Although it has been suggested that the main EHEC virulence genes (*stx*, *eae* and *ehx*) might be an advantage in survival and persistence of *E. coli*, the importance of these genes is still not fully understood. Most of the previous survival studies used non-pathogenic *E. coli* O157:H7 strains and the few survival data based on pathogenic EHEC O157 indicated that *stx1*, *stx2*, *eae* and *ehx* did not affect the survival of this pathogen (Nesse et al., 2014, Woodward et al., 2003). Sheng et al. (2006) demonstrated that *E. coli* O157:H7 missing these virulence factors were able to persist and be recovered from bovine terminal rectum, indicating that other factors are important for persistence of *E. coli* in the bovine GIT. However, examination of the effect of environmental stresses on virulence gene expression in *E. coli* O157:H7 using microarray analysis demonstrated the induction of *stx1a* under cold

shock conditions (Allen et al., 2008). Furthermore, conditions that induce the stringent response (nutrient stress response) led to stimulation of the locus of enterocyte effacement (LEE) genes regulator, Ler (Nakanishi et al., 2006). Additional virulence factors demonstrated to have role in survival to stress response were *ureC* or *ureD* which assist in surviving acidic environments (Steyert et al., 2011), *E. coli* attaching and effacing gene-positive conserved fragments (*ecf*) which has a role in the survival and persistence of *E. coli* in the environment and cattle (Yoon et al., 2005) as well as tellurite resistance bacteria (*ter*C, *terD*) could detoxify other molecules to survive oxidative stress and survive host macrophages.

1.7 Virulence traits

1.7.1 Shiga toxin and Stx phages

1.7.1.1 Shiga toxin

Some *E. coli* strains were recognised to produce bacteriophage-encoded cytotoxins similar to that of *Shigella dysenteriae* (Newland et al., 1985, O'Brien et al., 1984, O'Brien et al., 1982). These toxins were termed initially Verotoxin (VT) owing to their cytotoxic effect on Vero cells (Konowalchuk et al., 1977). Later, the term Shiga-like toxin was applied to acknowledge the genetic relationships of the toxin with that of *Shigella* (Strockbine et al., 1986). Most recently, the term Shiga toxin was adopted and *E. coli* isolates that harbour Stx are referred to as Shiga toxin-producing *E. coli* (STEC) (Paton and Paton, 1996). Based on the antigenic and genetic variations, two major types of Stx have been identified: Stx1 and Stx2, which has 60% sequence homology to Stx1 (O'Brien et al., 1984, Strockbine et al., 1986, Jackson et al., 1987). Stx1 and Stx2 are AB5-type toxins composed of B-pentamer

subunits that bind to glycolipid receptor Gb3 (Globotriaosylceramide) on the microvascular endothelial cell surfaces of the host, and a single enzymatically active A subunit which inhibits protein synthesis and causes apoptosis of endothelial cells (O'Brien et al., 1992, Bekassy et al., 2011). Amino acid substitutions within Stx in the StxA and StxB subunits may influence their biological properties and host specificity and as a result a number of variants exist within both Stx1 and Stx2 (Zhang et al., 2002a, Paton et al., 1995).

1.7.1.2 Shiga toxin subtypes

Shiga toxins are classified mainly into Stx1 and Stx2. Stx1 consists of the following subtypes: *stx1a, stx1c* and *stx*1d and *stx2* variants include *stx2c, stx2d, stx2e, stx2f, stx2g* (Scheutz et al., 2012) and *stx2dac*t which is activated by the intestinal mucus of humans or mice (Bunger et al., 2013). Some variants were identified to predict the risk of developing HC and/or HUS such as *Stx2c* (Friedrich et al., 2002, Orth et al., 2007) and *Stx2dact* (Bunger et al., 2013, Bielaszewska et al., 2006) whereas the strains expressing *stx1, stx1c, stx2d* or *stx2e* may associate with a milder illness or asymptomatic infections (Orth et al., 2007).

1.7.1.3 Stx phages

Stx bacteriophage (Stx phages), which have a role in transferring *stx* genes, are bacterial viruses that infect bacteria by injecting their DNA into susceptible hosts. Stx2 phages are closely related to lambdoid phages in relation to sequence level and gene organisation whereas Stx1 phages resemble lambda phage only across non-structural genes (Brussow et al., 2004). The Stx phages can be induced by a number of inducing agents that trigger the bacterial stress response known as SOS (regulated by RecA). Exposure of EHEC to SOS-inducing agents provokes the phage to enter the lytic cycle (Zhang et al., 2000, McGannon et al., 2010). Lytic cycle can lead to bacterial cell lysis causing increase in the free Stx that

either disseminate in the surrounding environment or will be able to infect phage-sensitive *E. coli* and undergo lysogenic infection (Cordovez et al., 1992). An example of inducing agents that cause bacteriophage induction (reviewed in (Kruger and Lucchesi, 2015)) are antimicrobials such as trimethoprim-sulfamethoxazole (SXT) and ciprofloxacin (CIP) which are used for treatment of diarrhoeal disease in children and adults respectively (Zhang et al., 2000) and mitomycin C(Otsuji et al., 1959), DNase colicins (Toshima et al., 2007), high temperature coupled with UV treatment , hydrogen peroxide (Iversen et al., 2015) and hydrostatic pressure (Aertsen et al., 2005), amino acid starvation (Nejman-Falenczyk et al., 2012), EDTA and citrate(Imamovic and Muniesa, 2012).

1.7.1.4 Shiga-toxin bacteriophage insertion site (SBI).

The Stx bacteriophage can integrate into the bacterial host genome using phage encoded integrases. A range of Stx bacteriophage insertion sites (SBIs) have been identified and although there does seem to be consistency in insertion site for particular serogroups, new SBI's are being identified on a frequent basis. SBIs that have been described in the literature for EHEC O26 include *wrbA, yehV, yecE, sbcB, Z2577, argW, prfC* and *torS-torT* intergenic region (Steyert et al., 2012); however, the SBI for EHEC O26 circulating in Australia and the status of these loci in *stx*-negative strains is yet to be identified. To date, Stx phages of *E. coli* O26 have only been found integrated at *yehV, yecE, wrbA* and *sbcB.* Additional loci that have not been targeted in previous studies but could be insertion sites for the Stx phage are *yagQ, argU, ybhC, serC, yccA, phoH, potC, ycfD, yciD, ydaO, rspB, btuC, yecA, ryeB, asnT, yfaT, glyU, pheU, leuX, pntB, proL, pheV* (Steyert et al., 2012).

1.7.2 *eae*

Jerse et al. (1990) identified a host cell adhesion gene known as *eae* which encodes a 94kDa outer membrane adhesin known as intimin that is necessary for formation of the A/E lesion. The intimin is located on 35kb LEE Pathogenicity Island (PI) (Shaw et al., 2005) which also contains type III secretion system (T3SS), regulators, chaperones, translocated and effector proteins (Jerse and Kaper, 1991) that contribute to the formation of the A/E lesion.

eae has a highly conserved 5′-terminal region and variable 3′-terminal regions (Blanco et al., 2004b). Targeting *eae* genes in the variable 3′ region by PCR differentiates intimin alleles into types and subtypes: α1, α2, β1, β2 (ξR/β2B), β3, γ1, γ2, δ (δ/β2O), ε1, ε2 (νR/ε2), ε3, ε4, ε5 (ξB), ζ, η1, η2, θ, ι1, ι2 (μR/ι2), κ, λ, μB, νB, ο, π, ρ and σ (Oswald et al., 2000, Adu-Bobie et al., 1998, Tarr and Whittam, 2002, Blanco et al., 2004b, Zhang et al., 2002b, Blanco et al., 2004c, Blanco et al., 2004a).

It was observed that some *eae* variants are preferentially associated with defined EPEC and EHEC serotypes. While the β1 intimin was mainly detected among strains belonging to serotype O26:H11, intimin γ1was found in all EHEC O157:H7 utilised in the studies (Blanco et al., 2004a, Adu-Bobie et al., 1998). It was also observed that different intimin subtypes may drive bacteria to different intestinal colonisation sites. For instance, replacing the intimin γ1 encoded *eae of* O157 EHEC with α intimin encoded *eae* affected the colonisation site and lesions spread from the colon to the small intestine in gnotobiotic piglets (Tzipori et al., 1995).

1.7.3 LEE encoded and non-LEE encoded effectors

Other LEE encoded effectors and non-LEE encoded effectors (carried on pathogenicity islands, bacteriophage or plasmids) and their functions are listed in Table 1.2.

Table 1-2. Function of LEE encoded and non-LEE encoded effectors

Effector	Function	Reference
LEE encoded TTSS effectors		
EspA	Role in adhesion, colonisation and proteins translocation-through the EspA filament that forms bridge between the bacterium and the host epithelial cell.	(Misyurina et al., 2010)
EspB	Actin accretion beneath the attached bacteria, contributes to microvillus effacing and inhibits phagocytosis.	(Iizumi et al., 2007)
EspD	Forms a pore that allows translocation of other effector proteins such as EspA and EspB into the host cells membrane.	(Wachter et al., 1999)
Tir	A receptor for intimin, requires for attachment, colonisation and organisation of actin beneath the attached bacteria.	(Misyurina et al., 2010)
EspG-G2	Alter the epithelial paracellular permeability and disrupt the microtubule network of intestinal epithelial cells.	(Clements et al., 2011)
EspH	Modulates the host cytoskeleton structures and stimulates the formation of pedestal structure in infected HeLa cells.	(Tu et al., 2003)
Map	Targets mitochondria, promotes colonisation, and requires for perturbation of the intestinal barrier function and disruption the tight junctions.	(Ritchie and Waldor, 2005)
Non-LEE encoded TTSS effectors		
TccP	Induces actin polymerisation beneath attaching bacteria leading to pedestal formation.	(Garmendia et al., 2004)
EspJ	Has antiphagocytic activity.	(Marches et al., 2008)
EspL2	Promotes colonisation, triggers condensation of bacterial colonies on host epithelial cells and modulates the host cell membrane morphology.	(Miyahara et al., 2009)
EspM	Promotes stress fibres, triggers mislocalisation of tight junction and alteration in the morphology and architecture of infected polarised	(Arbeloa et al., 2008)

	monolayers.	
NleH	Bacterial colonisation factor which inhibits apoptosis, induces activation of NF-kB and expression of TNF-α.	(Hemrajani et al., 2010)
NleA/ EspI	Suppression of proteins trafficking between the endoplasmic reticulum and Golgi by interactions of NleA with Sec24 and disruption of intestinal tight junction.	(Lee et al., 2008)
NleB	Has a role in colonisation, repression of NF-kB and suppression of IL8	(Misyurina et al., 2010)
NleC	Inhibits inflammatory response by inhibition of NF-kB, inhibits release of IL-8.	(Sham et al., 2011)
NleE	Represses TNFα by degradation of NF-kB, blocks migration of NF-kB to the nucleus and suppresses secretion of IL8.	(Nadler et al., 2010)
NleG	Modifies host ubiquitination	(Wu et al., 2010)
NleL	Modulates the formation of pedestal onto which the bacteria adhere.	(Piscatelli et al., 2011)
EspK	has role in secretion and sensitive genetic marker for identification of EHEC and EHEC related strains	(Delannoy et al., 2013b)
Z2099	Unknown function. Sensitive genetic marker for identification of EHEC and EHEC related strains	(Delannoy et al., 2013b)
Z2098		

1.7.4 *E. coli* plasmid pO26:H11

1.7.4.1 Enterohaemolysin (*ehxA*)

ehxA is virulence factor which is encoded by *ehxCABD* operon and belongs to pore-forming cytolysins of the repeats in toxin (RTX) family which is located on the *E. coli* 168-kb plasmid pO26-Vir (Toth et al., 1990, Murase et al., 2012, Beutin et al., 1989, Schmidt et al., 1994). *ehxA* is related to the chromosomal encoded α-haemolysin (HlyA), which is widely distributed among gram negative bacteria, in its genetic organisation and calcium ion

dependency (Welch, 1991, Murase et al., 2012, Schmidt et al., 1996). In contrast to *Hly*, whose haemolytic activity can be detected on blood agar containing unwashed blood after 3 hrs of culture at 37^{o}C, *ehxA* has haemolytic activity that can be detected only on blood agar plates containing wash erythrocytes (EHX plate) after overnight incubation of the culture at 37^{o}C (Beutin et al., 1989, Murase et al., 2012). The haemolytic activity of *ehxA* due to the formation of hydrophilic pores in the cell wall results in release of haem and haemoglobin, both of which provide EHEC with iron sources (Taneike et al., 2002). Schmidt et al. (1995) suggested that *ehxA* works synergistically with *stx* and LPS and contributes to pathogenicity of EHEC associated HUS. However, association of HUS with *ehxA* positive isolates has been identified in *stx*-deficient strains suggesting a direct role of *ehx* in the pathogenicity of EHEC-associated HUS (Aldick et al., 2007, Schmidt et al., 1999b). In addition, the effect of *ehxA* in *stx*-deficient *E. coli* O26 strains from five *stx*-negative and e*hx*-positive patients diagnosed with HUS was investigated and direct cytotoxicity of *EhxA* to the microvascular endothelial cell line was observed giving rise to speculation that the damage caused by *ehx* might mediate the microvascular thrombosis and result in HUS (Aldick et al., 2007). However, Schmidt et al. (1999b) and Aldick et al. (2007) did not test the hypothesis that *stx* could be lost during subculture or during infection in the human host which has been reported for EHEC from clinical source (Bielaszewska et al., 2007, Karch et al., 1992).

1.7.4.2 pO26-Vir additional virulence factors

In addition to *ehxA*, pO26-Vir carries several virulence factors which have been implicated in the pathogenicity of *E. coli*. These putative virulence factors include serine protease (*espP*) (Fratamico et al., 2011), catalase-peroxidase (*katP*) (Fratamico et al., 2011), adherence-conferring protein (*iha*) (Fratamico et al., 2011), a potential adhesin (*toxB*) (Fratamico et al., 2011) and *ecf* (Yoon et al., 2005).

1.8 Genotypic methods for characterisation of *E. coli* O26

Acquisition of new genes which are mediated by uptake of phage, plasmids and pathogenicity islands plays an important role in bacterial evolution, giving rise to new pathotypes, and affects bacterial pathogenicity. Therefore, further studies to identify genotypic characteristics of *E. coli* O26 pathotypes comparing strains of different pathotypes are required to identify virulence gene profiles and relationships between isolates of different pathotypes. A number of genotypic methods have been established to characterise *E. coli* O26 isolates. These methods are based on characterising the genetic components of bacterial strains such as presence or absence of genetic markers, sequence variation within a specific gene or even through the whole genome sequence (Norman et al., 2012). The selection of typing method depends on the purpose of the analysis, cost, available resources, desired discriminatory power and trained personnel (Table 1.3). PCR based characterisation are broadly used for identification of serotype and virulence genes while pulsed field gel electrophoresis (PFGE), multiple-locus variable number tandem repeat (MLVA) and multilocus sequence typing (MLST) are the most common informative methods for epidemiological surveillance and investigation of relationship between isolates. For instance, using PFGE and MLVA, *E. coli* O26 aEPEC and EHEC lineages could be identified (Miko et al., 2010). In addition, MLST assigned O26 isolates successfully into *stx1a* phylogenetically distinct groups defined by sequence type ST21 and *stx2a* comprised both phylogenetic groups ST21 and ST29 (Bielaszewska et al., 2013).

Whole genome sequencing (WGS) has been utilised in many research laboratories and public health authorities for outbreak surveillance, epidemiological investigation, and identification and characterisation of bacterial pathogens including *E. coli* O26. Data arising from WGS can be analysed easily using available databases such as the Centre for Genomic

Epidemiology (CGE) which has demonstrated success in typing pathogens, prediction of AMR phenotype and identification of virulence genes profile as well as interrogation of the phylogenetic relationship between isolates using *in silico* MLST, MLVA, pangenome analysis and SNP analysis. Using a number of case studies, Food and Agriculture Organisation (FAO) recommended that WGS can contribute to improving food safety management and accelerating responses of regulatory and policy making agencies (FAO and WHO, 2016). Data generated by WGS can be exploited for food safety regulation and public health action by identifying source of contamination and used to prevent further illness by taking early preventive measures which required food safety experts, clinicians and industry to share the acquired data. Typing methods relevant to the thesis are presented in Table 1.3.

Table 1-3. Genotypic methods for characterisation of *E. coli* O26

Method	Definition	Application	Additional Notes
Pulsed field gel electrophoresis (PFGE)	Restriction enzymes (e.g XbaI) used to cut genomic DNA into small fragments at specific site to generate DNA fingerprint for a bacterial isolate	Research and epidemiological investigation to track source of outbreak	Availability: Variable Procedure and Data Analysis: Easy to perform and relatively easy to analyse Discriminatory power: High
Multiple-locus variable number tandem repeat (MLVA)	PCR based amplification method to identify polymorphisms in variable number tandem repeat	Epidemiological investigation to track source of outbreak and identify relatedness between isolates	Availability: Variable Procedure and Data Analysis: Easy to perform and relatively easy to analyse Discriminatory power: High
Multilocus sequence typing (MLST)	Identify relationship between isolates by identifying sequence variation in a single gen of housekeeping genes (mostly seven) to generate unique allelic profile to assign an isolate to MLST type.	Understanding the phylogenetic and the evolutionary relationship between isolates	Availability: Variable Procedure and Data Analysis: relatively easy to perform and fair to analyse Discriminatory power: Good
Single nucleotide polymorphism (SNP) analysis	Identify variation within a specific gene sequence or even through the whole genome sequence	Understanding the Epidemiological investigation, phylogenetic and the evolutionary relationship between isolates	Availability: Reasonable Procedure and Data Analysis: requires access to bioinformatic support Discriminatory power: High
Shiga toxin-encoding bacteriophage insertion (SBI)	Identifying the insertion site of Shiga toxin-encoding bacteriophage using PCR reaction	Understanding the phylogenetic relationship among *E. coli* isolates	Availability: Reasonable Procedure and Data Analysis: Easy to perform and relatively easy to analyse Discriminatory power: Fair
Whole genome sequencing (WGS)	Extracting the genomic DNA to sequence the entire genome of an isolate which can be compared to a known reference to identify similarity and variation with a sequence or the whole genome	Investigate various characteristics of the sequenced organism including virulence profiles, plasmids profile SNP analysis….etc., allowing management of microbiological food safety issues and illnesses	Availability: Feasible in large institutions and research centres for research Procedure and Data Analysis: Easy to perform but relatively difficult to analyse "requires access to bioinformatic support" Discriminatory power: High (FAO and WHO, 2016)

Table adapted from (Rivas et al., 2015, Sabat et al., 2013)

1.9 **Conclusion**

Recently, EHEC O26 has received increasing attention due to its involvement in foodborne disease outbreaks worldwide. In addition, investigating and controlling this pathogen is crucial for Australian red meat exporters in maintaining access to markets such as the USA and any others that regulate for the presence of EHEC. In comparison to O157, relatively little is known about phenotypic and genotypic characteristics of O26 strains from clinical cases and cattle circulating in Australia. The ability of *E. coli* O26 to survive the antimicrobial intervention proposed in Australian food industry has not been investigated previously. Although a number of studies have been conducted to characterise the ability O157 and non-O157 to survive antimicrobial intervention, most of these studies were in the USA and it cannot be assumed that *E. coli* O26 from Australia will respond similarly to *E. coli* O157:H7 or other *E. coli* strains when exposed to antimicrobial agents. Therefore, it is of importance for food industry if a study utilises antimicrobial agents that are typically used in Australian industry and isolates that have been isolated from Australian cattle or clinical cases. In addition, gaps exist in the literature relating to the capacity of EHEC O26 to form biofilm on surfaces commonly used in food industry in comparison to other *E. coli* O26 pathotypes. Research on biofilm formation and factors that enhance or limit this phenotype have utilised relatively few strains of a serotype and compared EHEC O26 to other EHEC seropathotypes (Chen et al., 2013, Nesse et al., 2014, Vogeleer et al., 2015, Wang et al., 2012). In fact, *E. coli* O26 has been described as a dynamic or heterogeneous group whose members can undergo bidirectional conversion (Bielaszewska et al., 2007), therefore characterisation of other O26 pathotypes that have the capacity to convert to EHEC, together with EHEC in the same study might assist in identifying unique attributes for

EHEC in comparison to other pathotypes which subsequently could assist in designing approaches to control this pathogen. Furthermore, the genotypic characteristics of biofilm-forming and non-forming isolates from clinical cases and cattle isolates circulating in Australia have not been explored previously. Differences between studies in identifying factors that affect biofilm formation make comparisons between studies difficult for some factors that impact biofilm formation with much of these likely due to incompatibility between methods used. The bidirectional conversion between EHEC and other pathotypes suggests that some *stx*-negative isolates might be EHEC that have lost *stx* or EHEC derivatives that could acquire the Shiga-toxin prophage. As *stx* is the main virulence factors, characterising isolates that display EHEC virulence characteristics, but lack *stx* would be of interest to understand the importance of these isolates to food industry. Most importantly, pathogenic clones of *E. coli* O26 has been described worldwide (Bielaszewska et al., 2013, Delannoy et al., 2015, Krüger et al., 2015). However, no study has been undertaken to investigate the characteristics of *E. coli* O26 clone circulating in Australia. It is anticipated that these investigations will assist in identifying those *E. coli* O26 most capable of surviving a range of food processing interventions as well as a subset strains that harbor additional genotypic traits that are commonly associated with human disease. The phenotypic and the genotypic characterisation of Australian *E. coli* O26 may assist in providing critical information for developing efficient strategies to control this bacterial pathogen.

1.10 **Aims of this study**

The purpose of this thesis was to phenotypically and genotypically characterise *E. coli* O26 isolates from clinical and cattle sources in Australia. The specific aims of this study were to:

1. Characterise a collection of temporally and spatially separated Australian *E. coli* O26 isolates from clinical and cattle sources to identify a subset of isolates that can be used for subsequent assays (Chapter 2).
2. Determine the response of the subset of *E. coli* O26 isolates in planktonic form of growth to a range of antimicrobials and sanitisers (Chapter 2).
3. Determine the capacity of O26 and other pathotypes to form biofilms and explore the influence of pathotype, cells surface hydrophobicity, and phenotypic and genotypic attributes on biofilm formation (Chapter 3).
4. Determine the influence of biofilms on an isolate's sensitivity toward sanitisers (Chapter 3).
5. Characterise Shiga-toxin bacteriophage insertion sites for EHEC strains and explore the ability of *stx*-negative strains to acquire the bacteriophage and establish a lysogen (Chapter 4).
6. Sequence and subtype *E. coli* O26 isolates and conduct comparative genome fingerprinting to compare and contrast between clinical and cattle sourced strains and between pathotypes (Chapter 5).

Chapter 2

Survival Capabilities of *Escherichia coli* O26 Isolated from Cattle and Clinical Sources in Australia to Disinfectants, Acids and Antimicrobials

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The work presented in this chapter represents the peer reviewed publication

Lajhar SA, Brownlie J, Barlow R. Survival capabilities of Escherichia coli O26 isolated from cattle and clinical sources in Australia to disinfectants, acids and antimicrobials. *BMC Microbiol* 2017; **17**:47.

Contribution

SL, RB and JB conceived and designed the experiments; SL performed the experiments, acquired the data the data and drafted the manuscript. RB assisted in interpreting the data and critically revised the manuscript**.**

2.1 **Abstract**

Background: After *E. coli* O157, *E. coli* O26 is the second most prevalent enterohaemorrhagic *E. coli* (EHEC) serotype identified in cases of foodborne illness in Australia and throughout the world. *E. coli* O26 associated foodborne outbreaks have drawn attention to the survival capabilities of this organism in a range of environments. The aim of the present study was to assess the ability of *E. coli* O26 to survive the effects of disinfectants, acids and antimicrobials and investigate the possible influence of virulence genes in survival and persistence of *E. coli* O26 from clinical and cattle sources from Australia.

Results: Initial characterization indicated that *E. coli* O26 are a genetically diverse group that were shown to belong to a number of pathotypes. Overall, 86.4% of isolates were susceptible to all antimicrobials tested with no significant differences in resistance observed between pathotypes. A representative subset of isolates (n=40) were selected to determine their ability to survive disinfectants at proposed industry working concentrations and acid stress. Profoam, Kwiksan 22, and Topactive Des. were able to inhibit the growth of 100% of isolates. The remaining three disinfectants (12.5%Dairy Chlor, Envirosan and Maxifoam) were not effective against the subset of 40 *E. coli* O26. Finally, elevated MICs (1,024 to 4,096 µg/ml) of acetic, propionic, lactic, and citric acids were determined for the majority of the isolates (85%).

Conclusion: Australian *E. coli* O26 isolates belong to a range of pathotypes that harbor differing virulence markers. Despite this, their response to antimicrobials, disinfectants and acids is similar confirming that stress response appears unrelated to the presence of EHEC virulence markers. Notwithstanding, the tolerance to disinfectants and the elevated acid MICs for EHEC

and the other *E. coli* O26 pathotypes examined in this study may contribute to bacterial colonization on food contact surfaces and subsequent foodborne illness caused by this pathogen.

2.2 **Introduction**

Enterohaemorrhagic *Escherichia coli* (EHEC) strains have been associated with a number of foodborne outbreaks which have led to life threatening sequelae such as haemolytic uremic-syndrome (HUS) and haemolytic colitis (HC) (Vally et al., 2012, Guth et al., 2002, Brooks et al., 2005). Epidemiological surveillance indicates that *E. coli* of O157:H7 serotype is the most frequently encountered EHEC implicated in sporadic and outbreak cases of illness (Paton and Paton, 1998). However, other non-O157 serotypes such as O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28 and their non-motile forms have emerged and are now considered an important cause of human infection resulting in HUS (Delannoy et al., 2013b). Among the non-O157 serotypes, *E. coli* O26:H11 is one of the major serotypes of concern (Brooks et al., 2005, Gerber et al., 2002).

Although it is not completely understood which suite of bacterial virulence determinants are most necessary for *E. coli* O26 to cause disease in humans, isolates recovered from clinical samples typically possess Shiga toxins 1 or 2 or both (*stx1*, *stx2*) encoded by lambdoid bacteriophages, the *E. coli* attaching and effacing gene (*eae*) which is located on the LEE island and is necessary for bacterial colonisation of the gut and formation of the A/E lesion, and enterohaemolysin *(ehx*), a plasmid encoded virulence factor thought to work synergistically with *stx* and contribute to the pathogenicity of EHEC (Zhang et al., 2012, Van de Kar et al., 1992, Schmidt et al., 1995, Jerse et al., 1990). It has been recognised that cattle represent a major reservoir of *E. coli* O26 (Lynch et al., 2012, Barlow and Mellor, 2010, Blanco et al., 2003). Exposure to this pathogen can occur by a variety of routes including contact with animals on farms, consumption of contaminated meat, milk and its derivatives, water, spinach, sliced

watermelon, clover sprouts, blueberries and strawberries (Ethelberg et al., 2009, Hiruta et al., 2001, Hoshina et al., 2001, Lynch et al., 2012). The contamination of food products with either *stx* positive or *stx*-negative *E. coli* O26 strains has been reported previously (Allerberger et al., 2003, Murphy et al., 2007, De Schrijver et al., 2008, Buvens et al., 2011, Madic et al., 2011) and in some cases it has resulted in the recall of food products and a number of outbreaks (Ethelberg et al., 2009, FSIS, 2016, Gerber et al., 2002). In 2005, there was an outbreak in France due to consumption of unpasteurised cow cheese contaminated with EHEC O26 (Espié et al., 2006). In 2007, EHEC O26 infections occurred amongst consumers of ice cream produced from pasteurised milk made and sold at a farm in Belgium (De Schrijver et al., 2008). Additionally, multistate outbreaks of EHEC O26 infections in the USA have been also reported by the CDC in 2010, 2011, 2013 and 2015.

The detection of *E. coli* O26 isolates in various environments including food, processing equipment and food contact surfaces and identifying them as a causative agent for a number of foodborne outbreaks creates the need for implementing prevention strategies to control this pathogen. Food producers and processors can use a range of antimicrobial agents such as sanitisers and disinfectants to assist in controlling this organism. Resistance of *E. coli* strains recovered from various environments to a variety of antimicrobial agents has been reported (Fukushima et al., 1999, Kalchayanand et al., 2012, Škaloud et al., 2003, Beier et al., 2013). Variations in the response of different strains of *E. coli* to a range of disinfectants was demonstrated by Skaloud *et al.*, (2003) and Beier *et al.*, (2013) and suggest that the stress response of a range of *E. coli* strains may be highly variable. To date, most studies have focused on the survival capability of *E. coli* O157:H7 and minimal information is known about the response of *E. coli* O26 strains to these types of stressors. It cannot be assumed that *E. coli* O26

will respond similarly to *E. coli* O157:H7 or other *E. coli* strains when exposed to antimicrobial agents. We hypothesised that EHEC O26 have enhanced ability to persist and survive antimicrobial intervention in their planktonic state and that this consequently contributes to EHEC incidence and infection. Therefore, the aim of the present study was to assess the ability of *E. coli* O26 recovered from clinical cases and cattle sources to resist the effects of disinfectants, acids and antimicrobials in the planktonic state and investigate the possible association of virulence genes such as *stx* and *eae* with the survival of Australian *E. coli* O26 from clinical and cattle sources.

2.3 Materials and Methods

2.3.1 Bacterial strains

A total of 88 *E. coli* O26 isolates collected previously from clinical (10) and cattle (78) sources between 1995 and 2013 were utilised in this study. *E. coli* O26 isolates were initially selected with broad criteria, based on virulence profiles, time and source. Isolates stored at -80°C in protect bacterial preservers (Technical Service Consultants Ltd) were subcultured on tryptone soya agar (TSA; Oxoid, UK) and incubated at 37ºC overnight.

2.3.2 PCR detection

Whole cell suspensions were created by suspending a single colony in 200 µl sterile Milli-Q water and tested by PCR for the presence of *stx* (*stx*$_1$ and *stx*$_2$), *eae*, *ehx*, *eae* conserved fragment (*ecf*), and bundle forming pilus *(bfpA*), using the primers shown in Table 2.1. PCR master mix

containing 10X Dream Taq™ Buffer (Thermo Fisher Scientific, Australia), 250 mM dNTPs (Thermo Fisher Scientific, Australia), 0.02 mg/ml bovine serum Albumin (Sigma-Aldrich, USA), 12.5 pmol forward and reverse primer (GeneWorks, Australia) and 1.25 U Taq DNA polymerase (GeneWorks, Australia) was used. PCR products were subjected to gel electrophoresis on 2% agarose gels for 45 min at 100 V with resulting bands then visualised using a UV transilluminator. The anticipated amplicon size for each PCR product is shown in Table 2.1.

2.3.3 Detection of *rmlA* SNP using restriction fragment length polymorphism (RFLP)

A single nucleotide difference (G→T) at position 30 within *rmlA* has been shown to be associated with the presence of *stx* in *E. coli* isolates (Norman et al., 2012). Primers rmlA 30snp-F and rmlA 30snp-R were used to amplify a 484 bp portion of *rml*A (Table 2.1). Amplified PCR products were digested for 4 hours at 37^{o}C using the restriction enzyme *Aci*I. PCR products were subjected to gel electrophoresis on 2% agarose gels for 45 min at 100 V with resulting bands then visualised using a UV transilluminator. Isolates harbouring the *rml*A SNP yielded 10bp (not visible), 161bp and 313bp fragments whereas those isolates without the SNP yielded 10bp (not visible), 109, 161 and 204bp fragments.

Table 2-1. PCR Primer sequences used in this study

Primers	Sequence	Amplicon size	References
stx1-F	5'-ATAAATCGCCATTCGTTGACTAC-3'	180	(Paton and Paton, 1998)
stx1-R	5'-AGAACGCCCACTGAGATCATC-3'		
stx2-F	5'-GGCACTGTCTGAAACTGATCC-3'	255	(Paton and Paton, 1998)
stx2-R	5'-TCGCCAGTTATCTGACATTCTG-3'		
eae-F	5'-GACCCGGCACAAGCATAAGC-3'	284	(Paton and Paton, 1998)
eae-R	5'-CCACCTGCAGCAACAAGAGG-3'		
hlyA-F	5'-GCATCATCAAGC GTACGT TCC-3'	534	(Paton and Paton, 1998)
hlyA-R	5'-AATGAGCCAAGCTGGTTAAGC T-3'		
wzx O26-F	5'-CGCGACGGCAGAGAAAATT-3'	326	(Perelle et al., 2004)
wzx O26-R	5'-ACAATCCAACCGAACCAAAC-3'		This study
ecf-F	5'-TATCAGCACCAAAGAGCGGGAACA-3'	99	(Luedtke et al., 2014)
ecf-R	5'-CCCTTATGAAGAGCCAGTACTGAA-3'		
rmlA 30snp-F	5'-AAGTCGCAGGCTTGT-3'	484	This study
rmlA 30snp-R	5'-CGAAGACCCGCTAAC-3'		
BFPA300-F	5'-GGAAGTCAAATTCATGGG-3'	300	(Taniuchi et al., 2012)
BFPA300-R	5'-GGAATCAGACGCAGACTGGT-3'		

2.3.4 Antimicrobial resistance (AMR)

To determine the AMR phenotype of *E. coli* O26 isolates a custom susceptibility panel AUSVN2 (TREK Diagnostics, UK) designed specifically for testing Australian Gram-negative isolates was used. All plates were inoculated and assessed using the Sensititre system (TREK Diagnostics). Antimicrobials that were tested are cefazolin, cefotaxime, ceftiofur, amoxicillin / clavulanic acid, nalidixic acid, cefoxitin, ciprofloxacin, meropenem, ceftriaxone, gentamicin, ampicillin, trimethoprim / sulfamethoxazole, chloramphenicol, kanamycin, tetracycline and streptomycin. The Clinical and Laboratory Standards Institute (CLSI) criteria were utilised to identify antimicrobial resistance breakpoints when available; otherwise European Committee on Antimicrobial Susceptibility Testing (EUCAST) and National Antimicrobial Resistance

Monitoring System (NARMS) values were used. *E. coli* ATCC 25922 was used as a control strain.

2.3.5 Pulsed field gel electrophoresis (PFGE)

PFGE was performed using the standardised PulseNet protocol with chromosomal DNA of *Salmonella ser. Braenderup* H9812 digested with XbaI (Roche diagnostics, USA) used as a molecular size marker (Hunter et al., 2005, CDC, 2004). PFGE gels were analysed using BioNumerics V7.5 (Applied Maths, Belgium).

2.3.6 Disinfectants and acids susceptibility

A total of six disinfectants and four acids were evaluated for their antimicrobial efficacy against *E. coli* O26 isolates. The disinfectants and acids tested were Topactive DES (Ecolab Pty Ltd, Australia), Dairy Chlor 12.5% (Campbell Cleantec, Australia), Maxifoam (Ecolab Pty Ltd, Australia), Envirosan (Ecolab Pty Ltd, Australia), Profoam (Jasol Australia, Australia), Kwiksan 22 (Ecolab Pty Ltd, Australia), acetic acid (Sigma-Aldrich, Mexico), citric acid (Sigma-Aldrich, Japan), lactic acid (Sigma-Aldrich, Japan), and propionic acid (Sigma-Aldrich, Japan). Disinfectants tested in this study are approved for use in Australian food industries and food processing areas. Each disinfectant was tested at concentrations spanning the recommended working concentrations. The active components of each of the disinfectants are as follows: Topactive Des: hydrogen peroxide solution (<10%), acetic acid (<10%), amines, C12-16-alkyldimethyl (<10%), N-oxides (<10%), peracetic acid (<10%); Dairy Chlor: sodium hypochlorite (10-<30%), sodium hydroxide (<10%); Maxifoam: potassium hydroxide (<10%),

builder (<10%), alkaline salts (<10%), anionic surfactant (<10%), surfactants (<10%), hydrotrope (<10%), sodium hypochlorite (<10%) and scale inhibitors (<10%); Envirosan: dodecylbenzenesulfonic acid (<10%), propanoic acid, 2-hydroxy-,(s) (<10%); Profoam: quaternary ammonium compounds (0-5%), surfactants (10-30%); Kwiksan 22: quaternary ammonium compounds, benzyl-C8-18-alkyldimethyl, chlorides (10-<30%). Evaluation of the effectiveness of the antimicrobial activities of disinfectants and acids and subsequent determination of the susceptibility profiles were performed on polystyrene microtiter plate using broth microdilution method as described previously (Beier et al., 2005, Beier et al., 2008). Briefly, a single colony from each isolate was streaked onto TSA agar plates and grown for 18 to 24 hr at 37^{o}C. Working solutions for each disinfectant were prepared by diluting with Müller-Hinton broth (MHB; Oxoid, UK) then sterilising using a 0.45 µm syringe filtre (Sartorius Stedim Biotech GmbH, Germany). The working solutions were then two-fold serially diluted to achieve the test concentrations and pH for each concentration was determined. *E. coli* O157:H7 Sakai strain was used as a control for the survival of isolates to disinfectant challenge and acid challenge assay.

2.4 Results

2.4.1 Characterization of *E. coli* O26 by PCR and RFLP

A total of 88 isolates were screened for the presence of PCR gene targets: *stx*$_1$, *stx2*, *eae*, *ehx*, *ecf*, *bfp* and the *rml*A SNP. A summary of the PCR screening and characterization of clinical and cattle isolates is shown in Table 2.2. All of the 88 isolates were found to be negative for *stx*$_2$ and *bfp*. Based on the results of the PCRs four distinct groupings were formed and subsequently referred to here as pathotypes: EHEC, potential EHEC (pEHEC), atypical Enteropathogenic *E. coli* (aEPEC) and Non-toxigenic *E. coli* (NTEC). The EHEC group includes all clinical and 40 (51.2%) cattle isolates. Of the remaining cattle isolates, 33 (42.3%) were aEPEC, three (3.8%) were pEHEC, and two (2.6%) were NTEC.

Table 2-2. Prevalence of genetic markers in *E. coli O26* from clinical and cattle sources

Pathotypes	Virulence makers	No of isolates	Source
EHEC	*stx* (*stx*$_1$), *eae*, *ehx*, *ecf*, SNP within *rml*A	50	Clinical and cattle
pEHEC	*eae*, *ehx*, *ecf*, SNP within *rml*A	3	Cattle
aEPEC	*Eae*	33	Cattle
NTEC	Negative for all virulence markers tested	2	Cattle

2.4.2 Antimicrobial resistance (AMR)

A total of 88 isolates were assessed for their resistance to 17 antimicrobials. The distribution of minimum inhibitory concentrations (MICs) for each antimicrobial, concentrations tested and resistance breakpoints are presented in Table 2.3. Overall, there was a low level of resistance among the cattle isolates with 86.4% of all isolates susceptible to all antibiotics tested in this study. In total, 12 (13.6%) *E. coli* O26 isolates (10 EHEC and two aEPEC) exhibited resistance to at least one antimicrobial. Of the 12 isolates, four were resistant to only one antimicrobial with resistance to streptomycin or nalidixic acid observed in two and one EHEC isolates from cattle, respectively, and a single aEPEC isolate from cattle demonstrating resistance to tetracycline. Multidrug resistance was observed in three clinical and five cattle isolates. The most common co-resistance phenotype observed was ampicillin-kanamycin-streptomycin-tetracycline (one cattle EHEC strains and two clinical EHEC strains) and ampicillin-streptomycin (three cattle EHEC isolates) while resistance to both chloramphenicol-streptomycin and ampicillin-streptomycin-tetracycline were found in a clinical EHEC isolate and a cattle aEPEC isolate, respectively. Although AMR appeared largely constrained to EHEC isolates with 20% (10/50) demonstrating resistance to at least one antimicrobial, the differences in AMR prevalence between pathotypes was found to not be significant (p=0.05).

Table 2-3. Distribution of antimicrobial MICs among *E. coli* O26 isolates from cattle and human sources

Class	Antimicrobial (Abreviation)	Group n=88	% Resistant	95% CI	Range µg/ml	Concentration µg/ml												
						<0.03	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Aminoglycosides	Gentamicin (GEN)	Human=10			0.5-16					40		40	20						
		Cattle=78								60.3		39.7							
	Kanamycin (KAN)	Human=10	20	2.52-55.6	8-64									80					20
		Cattle=78	1.3	0.03-6.94										97.4		1.3			1.3
	Streptomycin (STR)	Human=10	30	6.67-65.2	16-64										70				30
		Cattle=78	8.9	3.9-17.6											91			1.3	7.6
b-lactam/b-lactamase inhibitor combinations	Amoxicillin/clavulanic Acid (AUG)	Human=10			1/0.5-32/16									80	20				
		Cattle=78											1.3	71.8	26.9				
Carbapenem	Meropenem (MERO)	Human=10			0.06-0.5		100												
		Cattle=78					100												
Cephems	Cefazolin (FAZ)	Human=10			2-16							80		20					
		Cattle=78										79.4		2.6					
	Cefotaxime (FOT)	Human=10			0.03-8	10		90											
		Cattle=78				10.2		83.3	6.4										
	Cefoxitin (FOX)	Human=10			0.5-32									100					
		Cattle=78											1.3	74.4	20.5	3.8			
	Ceftiofur (XNL)	Human=10			0.5-16					100									
		Cattle=78								97.4		2.6							
	Ceftriaxon (AXO)	Human=10			0.12-4			100											
		Cattle=78						96.1		1.3	1.3	1.3							
Folate pathway inhibitors	Trimethoprim/Sulfamethxazole (STX)	Human=10			2-32			70	10	20									
		Cattle=78						84.6	7.8	7.8									
Penicillins	Ampicillin (AMP)	Human=10	20	2.52-55.6	2-64									80					20
		Cattle=78	6.4	2.11-14								1.3		87.1	5.1				6.4
Phenicols	Chloramphenicol (CHL)	Human=10	10	0.25-44.5	2-32									20	70			10	
		Cattle=78												2.6	87.1	10.3			
	Florfenicol (FFN)[a]	Human=10			2-64									30	60				10
		Cattle=78												5.1	88.5	6.4			
Quinolones	Ciprofloxacin (CIP)	Human=10			0.06-4		100												
		Cattle=78					97.4		1.3	1.3									
	Nalidixic Acid (NAL)	Human=10			1-32								70	30					
		Cattle=78	1.3	0.03-6.94							1.3		52.6	44.9				1.3	
Tetracyclines	Tetracyclin (TET)	Human=10	20	2.52-55.6	2-16							80					20		
		Cattle=78	3.9	0.8-10.8								92.3		2.6	1.3	2.6	1.3		

[a] Only a susceptible breakpoint (≤4µg/ml) has been established. Isolates with an MIC ≥8µg/ml are reported as non-susceptible. Vertical lines indicate breakpoints for resistance. The white fields indicate the dilution range tested for each antimicrobial. Grey area indicates MIC values greater than or less than the tested concentration. Number of isolates is in percentage (%). * CI: Confidence intervals.

2.4.3 PFGE

Analysis of PFGE patterns revealed that the *E. coli* O26 strains in this study are highly diverse with similarity between isolates ranging from 71.4-100% (Figure 2.1). Comparison of PFGE profiles of 88 isolates identified 75 distinct PFGE patterns at a similarity cut-off level of 100%. At a cut-off value of 90%, isolates could be grouped into 40 clusters, of which, 19 PFGE clusters were represented by a single isolate and the remaining 69 isolates grouped into 21 clusters containing between two and nine isolates. Of the 69 isolates, 44 had distinct PFGE patterns with the remaining 25 isolates splitting into 11 clusters of 2 indistinguishable isolates and 1 cluster of 3 indistinguishable isolates. Of note, two isolates that were unrelated temporally (one from cattle and one from human) produced indistinguishable PFGE patterns. PFGE patterns were classified into two main clusters designated A and B at a similarity level of 74%. Cluster A included 49 (98%) of EHEC, two pEHEC and one aEPEC isolate. Isolates grouped in cluster B were 97% (32/33) aEPEC as well as two NTEC, a single pEHEC and a single EHEC isolate. An association between clusters and AMR isolates were not found (*P* value > 0.05). To capture the diversity of *E. coli* O26 strains, a subset of O26 isolates (n=40) representing various AMR profiles, a range of *E. coli* O26 pathotypes and a diverse PFGE pattern, were then chosen for subsequent tests.

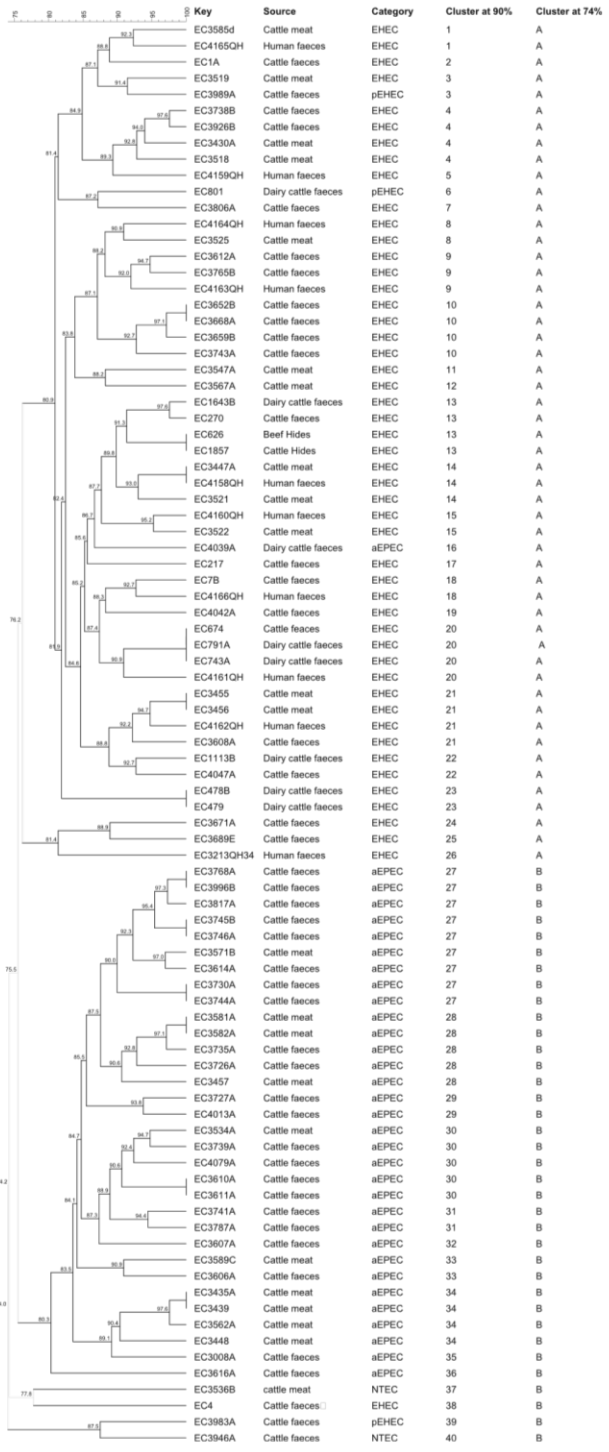

Figure 2-1. PFGE profiles and clusters of O26 isolates investigated in this study. All 88 isolates were analysed by PFGE with XbaI, and cluster analysis of the patterns was performed by BioNumerics V7.5 software using the Dice coefficient and unweighted pair group method.

2.4.4 Susceptibility to disinfectants

The effectiveness of disinfectants currently used in the food industry against 40 *E. coli* O26 was examined in this study and results are shown in Table 2.4. The proposed industry recommended concentrations for Kwiksan, Profoam, Topactive Des., Dairy Chlor 12.5%, Maxifoam and Envirosan were 0.45%, 1%, 1%, 0.3%, 1.6% and 0.4%, respectively. The most effective disinfectants with respect to their suggested working concentrations were Kwiksan 22, Topactive Des. and Profoam with each able to inhibit the growth of the strains tested with MICs at or below the working concentrations. Dairy Chlor 12.5%, Maxifoam and Envirosan were less effective against the 40 *E. coli* O26 examined in this study with all strains able to grow at a concentration at or above the suggested working concentrations. Importantly, *E. coli* O26 isolates missing any or all EHEC virulence markers (i.e. aEPEC, pEHEC and NTEC) were able to survive the same concentrations of disinfectant tested in our study against EHEC isolates, showing the same $MICs_{\%}$ as EHEC. When comparing the effective concentrations of disinfectants required for *E. coli* O26 isolates and the control strain *E. coli* O157:H7 Sakai, similar effectiveness was observed with elevated MICs demonstrated against Dairy Chlor 12.5%, (MIC= 2.4%), Maxifoam (MIC= 3.2%) and Envirosan (MIC= 1.6%). The remaining three disinfectants (Kwiksan 22, Topactive Des. and Profoam) that have shown to be effective against *E. coli* O26 were also effective against *E. coli* O157:H7 Sakai at the proposed industry working concentrations.

Table 2-4. Distribution of disinfectant MICs among 40 *E. coli* O26 isolates from cattle and human sources

Disinfectant	Range (%)	Concentrations (%)										
Topactive Des	0.0156-16	0.0156	0.0313	0.0625	0.125	0.25	0.5	**1**	2	4	8	16
					12 (30%)	28 (70%)						
Dairy Chlor	0.0188-12.5	0.01875	0.0375	0.075	0.15	**0.3**	0.6	1.2	2.4	4.8	9.6	12.5
									26 (65%	14 (35%)		
Maxifoam	0.025-25.6	0.025	0.05	0.1	0.2	0.4	0.8	**1.6**	3.2	6.4	12.8	25.6
									35 (78.5%)	5 (12.5%)		
Envirosan	0.025-25.6	0.025	0.05	0.1	0.2	0.4	0.8	1.6	**3.2**	6.4	12.8	25.6
									37 (92.5%)	3 (7.5%)		
Profoam	0.078-8	0.078	0.015	0.03	0.06	0.125	0.25	0.5	**1**	2	4	8
								39 (97.5%)	1 (2.5%)			
Kwiksan 22	0.0035-3.6	0.0035	0.007	0.014	0.028	0.056	0.112	0.225	**0.45**	0.9	1.8	3.6
				33 (82.5%)	7 (17.5%)							

Light Grey fields with bold fond indicate the recommended working concentrations for each disinfectant, Dark Grey fields indicate the dilution range tested for each disinfectant agents. Numbers in the white field indicates number and percentage of isolates susceptible to disinfectants at the tested concentration.

2.4.5 Susceptibility to acids

The MIC distribution profiles of 40 *E. coli* O26 isolates against four acids (acetic, propionic, lactic, and citric acids) are shown in Table 2.5. For comparison of MICs of *E. coli* O26 to acids with different molecular weights, the values for molar MICs ($MICs_{molar}$) were used. Based on Weight/Volume (w/v) concentrations it appears that the order of acids with the most effect on the *E. coli* O26 strains is acetic acid and propionic then lactic followed by citric acid with MICs of 1,024, 1024, 2,048, and 4,096 µg/ml, respectively (Table 2.5). Recalculation of the MICs to molar values reveals that propionic, acetic, citric and lactic acids have $MICs_{molar}$ of 13.82, 17.05, 21.3 and 22.7 mmole/ml, respectively, suggesting that propionic acid shows higher efficacy than acetic acid at retarding the growth of *E. coli* O26. When exposed to acids, the MICs for all 40 isolates occurred at an acetic acid pH and propionic acid pH that was much less acidic than that of the other two acids. The $MICs_{molar}$ for 87.5% (35/40) and 12.5% (5/40) of isolates occurred at an acetic acid pH 4.08 and 4.42, respectively. For propionic acid the $MICs_{molar}$ for 100% (40/40) of isolates occurred at pH of 4.55. When exposed to citric acid, the $MICs_{molar}$ for 87.5% (35/40) and 12.5% (5/40) of isolates were observed at citric acid pH of 3.75 and 3.31, respectively, whereas the $MICs_{molar}$ for 100% (40/40) of the isolates occurred at lactic acid pH of 3.67. No significant differences in susceptibility to acids was observed between pathotypes regardless of the type of acid assessed. Of note, the MICs of the tested *E. coli* O26 isolates in the acid challenge assay were comparable to the MIC values observed for the control strain of *E. coli* O157:H7 Sakai with MICs for acetic, citric, propionic and lactic acids of 512, 2048, 1024 and 2048 µg/mL observed.

Table 2-5. MICsμg/ml distribution of acids for 40 *E. coli* O26 isolates from clinical and cattle

Acids	Tested range (μg/ml)	MIC μg/ml	pH	No of isolates (%)
Acetic acid	64-65563	1024	4.08	35 (87.5)
		512	4.42	5 (12.5)
Citric acid	64-65563	4096	3.31	35 (87.5)
		2048	3.75	5 (12.5)
Lactic acid	64-65563	2048	3.67	40 (100)
Propionic acid	64-65563	1024	4.55	40 (100)

2.5 Discussion

O26 is the second most prevalent serotype identified in cases of foodborne illness attributed to *E. coli* in Australia and throughout the world. A range of measures exists for food producers to limit the spread and transfer of these organisms, however little is known about the variability of response to these control measures by *E. coli* O26 isolates. Isolates included in this study could be categorised into four pathotypes (EHEC, pEHEC, aEPEC and NTEC) based on the presence or absence of EHEC associated markers (*stx*, *eae*, *ehx*). Whilst isolates belonging to the EHEC group are of most interest because of their link to human clinical disease, this study identified a number of pEHEC organisms that appear to differ from EHEC isolates through the absence of *stx*. The ability of EHEC to acquire and lose *stx* has been described previously (Bielaszewska et al., 2007) and consequently there is a need to consider the clinical impact of these isolates.

The development of antimicrobial resistance within *E. coli* and particularly EHEC O26 remains an ongoing concern. In this study, a low level of antimicrobial resistance was observed with 86.4 % of isolates susceptible to all antimicrobials tested. These data are consistent with other studies that evaluated the AMR status of *E. coli* in Australian cattle populations at slaughter and in food purchased at retail (Barlow and Gobius, 2008, Barlow et al., 2015). These studies determined

that approximately >92% of isolates were susceptible to all antimicrobials tested. Furthermore, resistance to antimicrobials of critical or high importance in human medicine was not identified. As previously stated, comparison of the AMR results from different *E. coli* O26 pathotypes determined that although resistance was largely identified in EHEC isolates, these differences were not significant from any of the other pathotypes.

The use of PFGE enabled the identification of two distinct clusters at a similarity level of 75%. Cluster A was primarily composed of EHEC isolates and included a large proportion (98.7%) of isolates that were positive for the *ecf* and SNP within *rmlA* suggesting that these markers are notable features that could be used to define cluster A from cluster B. Interestingly, an individual aEPEC isolate grouped in PFGE cluster A and one EHEC isolate grouped into cluster B where the majority of isolates were aEPEC. This result may reinforce the hypothesis of emergence of EHEC and Non-EHEC by loss and gain of the stx gene. In fact, previous studies showed that conversion of EHEC O26 to *stx*-negative *E. coli* O26 is bidirectional where EHEC O26 lose *stx* genes converting to aEPEC and aEPEC O26 can be lysogenised with Stx-encoding phages to give rise to the emergence of EHEC (Bielaszewska et al., 2007). Another explanation could be that aEPEC isolates located in the same cluster with EHEC may contain pathogenic O island (OI-122, OI-43. OI-48, OI-50 or OI-57) encoded genes which were found to be significantly associated with aEPEC that showed high similarity to EHEC irrespective of their virulence attributes (Bugarel et al., 2011b, Delannoy et al., 2013a).

E. coli O26 strains that have been responsible for a number of foodborne outbreaks or isolated from a variety of food matrices and food producing animals draw attention to their tolerance to the environmental stresses applied in the food processing areas and food industry (Allerberger et

al., 2003, Hiruta et al., 2001, Madic et al., 2011, Wells et al., 1991). Subsequently, an evaluation for the effectiveness of the sanitisers used in the food processing environment, and food contact surfaces is crucial for understanding effective pathogen control. Both Profoam and Kwiksan are Quaternary Ammonium Compound (QACs) cationic surfactants that are widely used in clinical and industrial settings. Similarly, Topactive Des. which has peracetic acid as its active ingredient is used in the food industry and for disinfection of medical supplies. The observed MICs of *E. coli* O26 of different pathotypes to a range of QACs (Profoam and Kwiksan) and Topactive Des. demonstrated that the required MICs for inhibiting the growth of *E. coli* O26 carrying EHEC virulence markers (EHEC) and pathotypes lacking EHEC virulence factors (aEPEC, pEHEC and NTEC) are similar to that for *E. coli* O157 Sakai strain used as a control in our study. This suggests that the manufacturers' recommended concentrations for the tested disinfectants validated for O157 strain are effective for the control of *E. coli* O26 of various pathotypes and are may indeed be effective for most *E. coli* regardless of pathotypes or serogroup.

When challenged for their capability to survive disinfectants with sodium hypochlorite as the main component, both clinical and cattle *E. coli* O26 isolates of different pathotypes achieved MICs that exceeded the application concentrations. Consequently all forty isolates regardless of their pathotype were deemed to be non-susceptible to the recommended concentrations of Maxifoam and Dairy Chlor 12.5%. Pathogens that survive recommended concentrations of tested disinfectants pose a greater risk of spreading into the food supply chain and subsequently could contribute to the incidence of human disease thereby reinforcing the importance of continued evaluation of disinfectants. Škaloud *et al.*,(2003) reported that the MIC of sodium hypochlorite for both STEC O157 and O26 was 0.5% which is lower than the effective concentrations for disinfection of *E. coli* O26 in this study. Although these data may suggest variations among *E.*

coli strains in response to disinfectant stress comparison of the current results with others is difficult since the chosen susceptibility method is different. Previous studies raised concerns about the use of disinfectants and developing resistance to antimicrobial agents (Sidhu et al., 2002, Beier et al., 2013, Beier et al., 2005). These studies suggested that the use of disinfectants may impose selective pressure giving rise to the emergence of cross-resistance and co-resistance for widely used disinfectants and antimicrobial agents. In our study, the percentage of resistance to a range of disinfectants was similar among O26 isolates regardless of their AMR status and no association between the use of disinfectants and development of resistance to antimicrobial agents was found suggesting that the presence of either resistance has not resulted in selection for the other.

Organic acids have been used in foods as preservatives to enhance microbial safety. In addition, acids may be used as interventions in the beef industry to reduce bacterial contamination. Results presented here show that the majority of isolates (87%), regardless of their source and pathotypes, exhibited elevated MICs ($\geq$ 1024 μg/ml) to the tested acids. A previous study on the influence of organic acids on *E. coli* O157:H7 demonstrated elevated MICs for those acids as well (Beier et al., 2013). In addition, the MICs for the *E. coli* O26 isolates in this study occurred at low pH (4.55-3.31). Molina *et al*., (2005) have shown that STEC O26 and other STEC serotypes (O91:H2, O111:H$^-$, O145: H$^-$, and O157:H7) did not grow when they were exposed to citric acid and acetic acid at a pH of 4.5. Others reported that treatment with lactic acid at a concentration of 4%, reduced non-O157 including *E. coli* O26 by 2.3 log (Kalchayanand et al., 2012). Findings from the current and previous studies suggest that *E. coli* O26 isolates of different pathotypes utilise a number of acid resistance mechanisms to prevent the lethal effect of acidic stresses. The capacity of *E. coli* strains to withstand acidic environments during passage

and growth of these bacteria in the intestinal tracts of cattle and human and in acidic food is an important factor that influence their ability to survive and subsequently cause disease (Arnold et al., 2001).

2.6 **Conclusion**

In conclusion, *E. coli* O26 isolated in Australia are a genetically diverse group of organisms that belong to a range of pathotypes. The low level of resistance and the absence of AMR to clinically relevant antimicrobials in Australian cattle bacterial isolates are reflective of the comprehensive controls over the use of antimicrobials in food-production animals in Australia. However, the tolerance of EHEC and *stx*-negative *E. coli* O26 pathotypes (i.e. aEPEC, pEHEC and NTEC) to three of the tested disinfectants (Maxifoam and Dairy Chlor 12.5% and Envirosan) and the elevated $MICs_{\mu g/ml}$ to the acids examined in this study might contribute to bacterial colonisation of food contact surfaces, which may result in product contamination and subsequently foodborne illness. The ability of *E. coli* O26 isolates to survive a stress intervention was not related to a specific pathotype as isolates lacking EHEC associated markers such as *stx* or *eae* persisted at the same proportion as EHEC strains suggesting that other factors affect persistence of *E. coli* O26 strains. Knowledge of the virulence factors and genetic relatedness of *E. coli* O26 may improve our understanding of the capability of *E. coli* O26 to survive stress and subsequently cause human illness. Continuous evaluation of disinfectants and acids for their efficacy in reducing *E. coli* O26 should be conducted by food industries to assist in ensuring *E. coli* O26 is limited in its capacity to persist in food processing environments and contribute to foodborne disease.

Chapter 3

Characterization of Biofilm-Forming Capacity and Resistance to Sanitizers of a Range of *E. coli* O26 Pathotypes from Clinical Cases and Cattle in Australia

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Contribution

SL, RB and JB conceived and designed the experiments; SL performed the experiments, acquired the data, interpreted the data and drafted the manuscript. RB assisted in interpreting the data and critically revised the manuscript.

3.1 **Abstract**

Background: The formation of biofilms and subsequent encasement of bacterial cells in a complex matrix can enhance resistance to antimicrobials and sterilizing agents making these organisms difficult to eradicate and control. The aim of this study was to evaluate and compare the capacity of 40 *E. coli* O26 isolates of enterohaemorrhagic *E. coli* (EHEC, n=27), potential EHEC (pEHEC, n=3), atypical enteropathogenic *E. coli* (aEPEC, n=8) and non-toxigenic *E. coli* (NTEC, n=2) from clinical and cattle sources to form biofilms on different surfaces, and determine whether extracellular matrix (ECM) components (cellulose, curli), motility, prophage insertion in *mlrA* and cell surface hydrophobicity could influence biofilm formation. Finally, the influence of biofilm formation on the sensitivity of isolates to quaternary ammonium compounds (QACs; Profoam, Kwiksan 22) and peracetic acid-based sanitiser (Topactive Des.) for 2 min on polystyrene plate were also evaluated.

Results: Biofilm production on one surface may not indicate biofilm formation on a different surface. Biofilm was formed by different pathotypes on polystyrene (70%), stainless steel (87.5%) and glass slides (95%), however only 50% demonstrated pellicle formation. EHEC isolates were significantly more likely to form a pellicle at the air-liquid interface and biofilms on polystyrene surface at48 hours than aEPEC. Strains that don't produce ECM (curli or cellulose), harbor a prophage insertion in *mlrA*, and are non-motile, have lower biofilm forming capacities than motile strains that express ECM morphotypes and have intact *mlrA*. Hydrophobicity had no impact on biofilm formation. After 2 min exposure, none of the

disinfectants tested were able to completely inactivate all cells within a biofilm regardless of pathotypes and the amount of biofilm formed.

Conclusion: Pathotypes of *E. coli* O26 showed varying capacities to form biofilms, however, most EHEC strains had the capacity to form biofilm on all surfaces and the air-liquid interface and required only 48 hr to develop a biofilm. Biofilms provided a protective effect to *E. coli* O26 strains against the three sanitisers, previously shown to successfully control the growth of their planktonic counterparts. Whether the characteristics of biofilm forming and non-biofilm forming strains observed in this study reflect their attributes within the food-processing environment is unknown. Further studies that represent the conditions of food and meat processing environments are required.

3.2 **Introduction**

Enterohaemorrhagic *E. coli* (EHEC) are foodborne pathogens that have been implicated in a number of outbreaks with symptoms ranging from diarrhoea to haemolytic uremic syndrome (HUS) which can lead to death. Although O157 is the most common serogroup associated with foodborne disease, a number of other serogroups such as O26, O45, O103, O111, O121 and O145 are now considered as major causes of foodborne illness worldwide (Brooks et al., 2005, Gerber et al., 2002, Tozzi et al., 2003). Food producing animals, particularly cattle have been identified as a major reservoir of these pathogens and there have been several outbreaks attributed to the consumption of contaminated meat and milk products (Allerberger et al., 2003, Buvens et al., 2011, Ethelberg et al., 2009). Furthermore, in the United States of America (USA), EHEC of serogroup O157:H7 (USDA and FSIS, 1999) and additional six serogroups of O26, O45, O103, O111, O121 and O145 (USDA and FSIS, 2012) are considered adulterants of both raw, non-intact beef products such as ground beef, veal patties, and beef patties mix, and intact beef cuts that are to be further processed into non-intact cuts such as manufacturing trimmings of meat remaining after steaks or roasts are removed. Although the number of sporadic and outbreak cases of EHEC disease in Australia remains low, (Vally et al., 2012) EHEC are of economic importance as the Australian cattle industry is a significant exporter of red meat products. Investigating and controlling these pathogens is crucial in maintaining access to markets such as the USA and any others that regulate for the presence of EHEC. Among EHEC, *E. coli* O26 is one of the most common non-O157 serogroups associated with serious foodborne outbreaks worldwide (Ethelberg et al., 2004, Ethelberg et al., 2009, Gilmour et al., 2005, Madic

et al., 2011, Sonoda et al., 2008, Vally et al., 2012) with a number of food outbreaks linked to consumption of beef products and cattle dairy products (Allerberger et al., 2003, Buvens et al., 2011, Ethelberg et al., 2009, Madic et al., 2011). In Australia, EHEC, serogroup O26, can be isolated from clinical cases (Vally et al., 2012) and beef and dairy cattle (Barlow and Mellor, 2010, Mellor et al., 2016) albeit the prevalence in cattle populations and annual notification rate of EHEC O26-associated disease appears to be low when compared to other countries (Mellor et al., 2016, Vally et al., 2012). Nonetheless, as EHEC illness can lead to life-threating disease such as HUS, the presence of this organism represents a growing concern to the public health authorities and Australian red meat exporters and subsequently there is a need to understand how these organisms persist and transfer into farm-to-fork production chain.

Foodborne pathogens such as *E. coli* use a range of strategies to survive and persist in the environment. It has been shown that various *E. coli* serogroups including *E. coli* O26 have the capacity to form biofilms (Nesse et al., 2014, Vogeleer et al., 2015, Wang et al., 2012). The formation of biofilms and subsequent encasement of bacterial cells in a complex matrix and extracellular DNA (eDNA) can enhance metabolic capacity of cells within biofilm and provide protection against antimicrobials and sterilizing agents making these organisms difficult to eradicate and control (Uhlich et al., 2006, Wang et al., 2012, Vogeleer et al., 2015). Several factors have been demonstrated to affect biofilm formation including expression of extracellular matrix components (ECM; curli and/or cellulose), bacterial cell wall composition, temperature, hydrophobicity, surface charge, surface properties, pH, eDNA formation, QS-based extracellular cell–cell-signalling systems, organic residues on food surface and interaction with multispecies within biofilm communities (Vogeleer et al., 2015, Ogasawara et al., 2010, Burmolle et al., 2006, Bokranz et al., 2005).

Cellulose and curli which are one of the main structures in the biofilm matrix are regulated by DNA-binding transcription factor CsgD (Ogasawara et al., 2010). CsgD regulation requires protein transcription factors (Ogasawara et al., 2010) allowing *csgD* expression to be triggered by a number of stimuli such as temperature, pH, oxygen concentration, osmolality, and various nutrients (Uhlich et al., 2014). Recent studies also demonstrated that insertion of a prophage in *mlrA* (renamed from *yehV*) can negatively influence biofilm formation by reducing the *csgD* expression and subsequently acts as a barrier that limits curli expression and biofilm formation (Uhlich et al., 2013, Chen et al., 2013). The sigma factors RpoS and RpoD participate in the transcription of *mlrA* (Ogasawara et al., 2010) which is induced in the stationary phase.

The role of biofilm formation in human infection and contamination of food products has been well investigated (Carpentier and Cerf, 1993, Kumar and Anand, 1998). It has been suggested that biofilms in food-producing facilities act as a source of bacteria that may contaminate food products causing human infections and severe illness (Carpentier and Cerf, 1993, Kumar and Anand, 1998). In fact, after biofilm maturation, bacteria detach as a clump or planktonic cells as a result of internal biofilm factors (such as starvation and enzymatic degradation of the extracellular matrix) or external factors caused by application of physical methods (scrubbing or scraping) or breaking down the attachment forces (Stoodley et al., 2001, Fux et al., 2004). The detachment process allows bacteria to colonize new niches and maintain access to a nutrient-rich environment (Stoodley et al., 2001, Fux et al., 2004). Moreover, biofilm can offer an environment for horizontal gene transfer between members of biofilm communities. For instance, has been shown that dissemination of Stx-encoding bacteriophages can occur within biofilms and potentially enable the emergence of new *E. coli* pathotypes (Solheim et al., 2013).

A number of studies have investigated the biofilm forming capacity of non-O157 serogroups including EHEC O26, and the effectiveness of disinfectant interventions in restricting the growth of biofilms (Wang et al., 2012, Vogeleer et al., 2015). Whilst these studies gave insight into the protective effect of biofilms, it is important to understand whether the survival of pathogens to disinfectants differs depending on whether the cells are in a planktonic or biofilm state. Furthermore, it is of greatest relevance to the Australian food industry if a study utilises disinfectants that are typically used in industry and isolates that have been isolated from Australian cattle or clinical cases. Here, we hypothesised that EHEC O26 in their biofilm state had greater capacity to survive disinfectants than their planktonic counterparts, previously shown to be susceptible to disinfectants and this could consequently enhance its ability to transfer to humans via the food chain and subsequently cause human illness. Therefore, the aim of this study was to evaluate and compare the capacity of *E. coli* O26 isolates from clinical and cattle sources to form biofilm on different surfaces, and determine the association of biofilm with pathotypes, ECM components (cellulose, curli), motility, prophage insertion in *mlrA* and bacterial adhesion to hydrocarbons. Finally, the influence of biofilms on a strain's sensitivity toward the three sanitisers previously shown (Chapter 2) to be effective against their planktonic counterparts was also investigated.

3.3 Materials and methods

3.3.1 Bacterial strains

A total of 40 Australian clinical and cattle sourced *E. coli* O26 strains previously shown to represent the genetic diversity of Australian isolates were selected. The strains were selected

from a collection of 88 isolates based on their initial characterization (Chapter 2) by pulsed-field gel electrophoresis (PFGE), PCR for *stx, eae, ehx, bfp, ecf* and a single nucleotide polymorphism within *rmlA* along with their antimicrobial susceptibility and survival capabilities to disinfectants and acid approved for use in Australian food industry. Based on the presence or absence of *stx1*, *eae*, *ehx*, *ecf*, *bfp*, *rmlA* SNP strains were assigned into four pathotypes. Cattle strains were comprised of four pathotypes: enterohemorrhagic *E. coli* (EHEC; n=27), atypical enteropathogenic *E. coli* (aEPEC; n=8), non-toxigenic *E. coli* (NTEC; n=2) and potential EHEC (pEHEC; n=3; representing 30 distinguishable PFGE profiles while clinical strains were all EHEC O26 (n = 10) and represented 10 distinguishable PFGE profiles. In addition, all strains had wild type RpoS except two clinical strains (EC4164QH7 and EC4165QH8) which had mutation in RpoS (data not published).

3.3.2 Detection of curli and cellulose on Congo Red Indicator (CRI) agar

Curli and cellulose production was assessed on Congo Red Indicator (CRI) agar containing low salt (5 g/L) Luria-Bertani broth (LS-LB) supplemented with 40 mg/L of Congo red (Sigma, USA) and 20 mg/L brilliant blue (Sigma Aldrich, USA). Bacterial strains were initially cultured on LB agar (Oxoid; UK) and a single colony was inoculated into LS-LB broth and incubated statically for 18 hr at 37°C. An aliquot of 30 µl was spotted on CRI agar and incubated for 24 hr at 37°C, 48 hr at 30°C or 72 hr at 25°C. Expression of ECM components was determined based on colony morphology (RDAR: red, dry and rough colonies, express curli fimbriae and cellulose, PDAR: pink, dry and rough colonies, express cellulose, BDAR: brown, dry and rough colonies, express curli fimbriae and SAW: smooth and white colonies, do not express curli or cellulose morphotype) (Bokranz et al., 2005).

3.3.3 Motility

Strains were tested for motility in standard motility agar containing 3 g/L agar. Motility was investigated after 48 hr at 25°C. Non motile strains were re-examined each 24 hr for up to 7 days. Strains that did not show motility in 3 g/L agar were subsequently passaged up to three times in fresh low-percentage-motility media containing 2 g/L agar in an effort to induce motility. Each strain was examined in triplicate.

3.3.4 Prophage insertion in *mlrA*

To identify whether a prophage is inserted in the *mlrA*, all strains were screened by PCR using each of primer sets *yehV*-attB (Primers A and B) (A: AAGTGGCGTTGCTTTGTGAT and B: ACAGATGTGTGGT GAGTGTCTG) and *yehV*-attL (Primers F and B), (F: CACCGGAAGGACAATTCATC, B: AACAGATG TGTGGTGAGTGTCTG) (Shaikh and Tarr, 2003). The PCR PCR amplification reaction contained 2 µl of boil cell lysate and 23 µl of master mix that consisted of 10X Dream Taq™ Buffer (Thermo Fisher Scientific, Australia), 250 mM dNTPs (Thermo Fisher Scientific, Australia), 0.02 mg/ml bovine serum Albumin (Sigma-Aldrich, USA), 12.5 pmol forward and reverse primer (GeneWorks, Australia) and 1.25 U Taq DNA polymerase (GeneWorks, Australia). The PCR conditions used were 94°C for 5 min, followed by 30 s at 94°C, 30 s at 62°C, and 60 s at 72°C for 30 cycles and finally 72°C for 5 min. Amplified PCR products were analysed by gel electrophoresis, stained with ethidium bromide and the bands were visualised with UV transilluminator. Using the F/B primer pair, amplification of a 702 bp DNA product is expected when a prophage is inserted in the *mlrA* loci

(interrupted *mlrA* loci); when no prophage inserted in *mlrA* (intact loci), a 340 bp products is expected to be amplified using primers A/B.

3.3.5 Cell surface hydrophobicity

Cell surface hydrophobicity was measured using the bacterial adhesion to hydrocarbons (BATH) assay as described previously using xylene (Reagent Plus, 99%; Sigma Aldrich, USA) (Rivas et al., 2005) and hexadecane (Reagent Plus, 99%; Sigma Aldrich, USA) (Nesse et al., 2014). The test was performed at 25°C (48 hr incubation) and 37°C (24 hr incubation). Following incubation, a 1 ml aliquot of the lower aqueous layer was gently aspirated and the OD_{600} was measured. All OD measurements were determined using Novaspec II spectrophotometer (Pharmacia Biotech Ltd, UK). The percentage of bound cells to hydrocarbon for each strain was calculated according to the following formula: [(OD_{600} untreated bacterial cells - OD_{600} aqueous phase)/ OD_{600} untreated bacterial cells]*100.

3.3.6 Biofilm formation on polystyrene microtiter plates

Assessment of biofilm formation on polystyrene plates at 24, 48 or 72 hr at 25˚C without shaking was performed as described previously (Wang et al., 2012). In brief, cultures were prepared by initially inoculating a single colony into LS-LB broth and incubating for 18 hr at 37˚C with shaking at 150 rpm to reach a cell concentration of 8 $\log_{10}$ CFU/ml. The resulting enrichment was 100-fold diluted in sterile LS-LB and added to 96-well flat-bottom polystyrene plates (Sarstedt, USA) at 200 µl per well. Plates were incubated for 24, 48 or 72 hr at 25˚C without shaking. Following incubation, the bacterial suspension was removed and plates were washed in triplicate with 270 µl sterilised phosphate-buffered saline (PBS; pH 7.2) to remove unattached or

loosely attached cells. The plates were then air dried and stained with 100 µl per well of 0.1% crystal violet (CV) for 20 min. The plates were washed three times with PBS to remove excess stain, air dried and then 100 µl per well of 85% ethanol was added to each well to dissolve CV. Absorbance of the samples (As) were measured at optical density (OD_{570}) using a microplate reader (EnSpire® Multimode Plate Reader-PerkinElmer, USA) and the amount of biofilm formation was assessed by subtracting the mean of parallel assays from the average absorbance of the negative control (Ac). Three biological replicates were performed, each containing six technical replicates well per strain. Based on the OD produced by bacterial biofilms at 570 nm, strains were classified into the following categories as previously described (Stepanovic et al., 2004): As ≤ Ac = no biofilm producer, Ac < As ≤ (2 x Ac) = low biofilm producer, (2 x Ac) < As ≤ (4 x Ac) = moderate biofilm producer and (4 x Ac) < As= strong biofilm producer. Sterile LB broth was used as a negative control and *Salmonella typhimurium* strain ATCC 14028 was used as a positive control in all biofilm experiments as it is known to produce RDAR at 28˚C but SAW at 37˚C (Romling et al., 1998).

3.3.7 Biofilm formation on stainless steel and glass slides

Stainless steel coupons (0.9 mm thickness, size 50 x 20 mm) were prepared by being soaked in acetone for 30 min to remove contaminants and rinsed in water prior to soaking in 1 N NaOH for one hr. After soaking in 1 N NaOH, the stainless steel coupons were rinsed with at least 5 L of distilled water and sterilised by autoclaving. No pre-treatment of glass slides, other than autoclaving, was performed and slides were used as manufactured. One ml aliquots of overnight culture (approximately 8 $\log_{10}$ CFU/ml) were inoculated into 50 ml sterile Röhre tubes (Sarstedt, Germany) containing 9 ml of LS-LB. A sterile glass slide (76 by 26 mm; Menzel GmbH+CoKG,

Braunschweig, Germany) or a sterile stainless steel coupon was placed in each tube and only partially submerged in the broth to have an atmospheric interface with the liquid. The tubes were incubated at 25˚C for 72 hr without shaking. After incubation, the slides/coupons were washed with water and then transferred to a test tube with 1% CV solution for staining of the biofilm for 20 min. Excess CV solution was rinsed from the slides/coupons using water. Sterile LB broth was used as a negative control. Surfaces were then examined visually and given scores ranging from 0 (no visible biofilm) to 3 (thick biofilm at the air-liquid interface) according to the amount of stained biofilm observed (Nesse et al., 2014).

3.3.8 Pellicle formation at the air-liquid interface

Assessment of pellicle formation at the air-liquid interface was based on the CV staining assay in glass tubes as described previously (Wang et al., 2012). Approximately 8 $\log_{10}$ CFU/ml culture were diluted 100 fold and added at 2 ml per glass tube and incubated at 25˚C for 5 days without shaking. At the end of the incubation period, supernatants were gently removed, and all tubes were washed with 3 ml per tube of PBS then allowed to dry at room temp. Tubes were then stained with 3 ml per tube of 0.1% CV for 20 min at 22 to 25˚C, washed twice with 3 ml per tube of PBS, air dried again and subsequently assessed visually for pellicle formation. Strains were considered positive when the top surface of the culture was covered with an opaque pellicle layer attached to the wall of the tube. Quantitative measurement was performed by dissolving CV stained pellicle in 4 ml of 85% ethanol and the $O.D_{570}$ was measured using microplate reader at 200 µl per well.

3.3.9 Tolerance of *E. coli* O26 biofilms to disinfectants

The protective effect of biofilm on strains was assessed by exposing biofilm to quaternary ammonium compounds (QACs; Profoam, Kwiksan 22) and peracetic acid-based sanitiser (Topactive Des.) for 2 min. All strains that demonstrated the capacity to form biofilm on polystyrene plate at 24, 48 or 72 hr at 25˚C were assessed. Strains were allowed to form biofilm on polystyrene plate as outlined above. At the end of the incubation period, bacterial supernatants were gently aspirated and discarded, and each well was washed in triplicate with 200 µl of sterile PBS. The plates were dried and 200 µl of sterile PBS was added to three wells as an untreated control, while another three wells were filled with 200 µl of either 1% Profoam, 0.45% Kwiksan 22 or 1% Topactive Des. and incubated for 2 min at 25°C. At the end of the exposure time, antimicrobial agents were removed by aspiration and 170 µl of sterile Dey Engley broth (DEB; BBL, Difco, Sparks, MD) supplemented with 0.3% soytone and 0.25% sodium chloride was added to each well to neutralise the effect of disinfectants. The surface of each well was then scraped with sterile pipette tips and the contents transferred into a sterile tube which then vigorously vortexed to dislodge cell aggregates. The bacterial biofilm cells in the tubes were diluted, vortexed and subcultured on nutrient agar for enumeration of viable cells. Three biological replicates were performed for each strain with PBS and disinfectants.

3.4 Statistical Analysis

Calculation of linear correlation between two variables and one way analysis of means (Tukey's method) was performed using Minitab software (Minitab 16; Minitab Inc., Minneapolis, Minn). A *P* value of equal to or less than 0.01 was considered significant.

3.5 **Results**

3.5.1 Detection of curli and cellulose on CRI agar

Colony morphotypes (curli, cellulose, none) were assessed on (CRI) agar plates, and representative morphotypes are shown in Figure 3.1. Of the 40 *E.coli* O26 strains assessed, 22 (55%) strains demonstrated ability to produce at least one of the ECM components (Table 3.1). Regardless of the growth conditions, the ability to exhibit the BDAR morphotype (curli expression) was seldom observed with just two NTEC strains, one pEHEC and one EHEC displaying this morphology. The PDAR morphotype was more regularly observed with 16 (59.3%) EHEC and two pEHEC strains exhibiting this morphology. RDAR morphotype does not seem to be a common characteristic of *E. coli* O26 strains as only a single EHEC strain expressed both cellulose and curli and only at 37˚C. aEPEC strains (100%) were characterised by the expression of SAW morphotypes at 25, 30 and 37˚C. In contrast, only eight EHEC strains (29.6%) expressed SAW morphotype at all tested temperatures. Comparing EHEC from clinical and cattle sources, three clinical strains (30%) and five cattle strains (29.4%) showed SAW at 37°C, 30°C and 25°C and two clinical strains showed BDAR at 37°C but SAW at 30°C and 25°C.

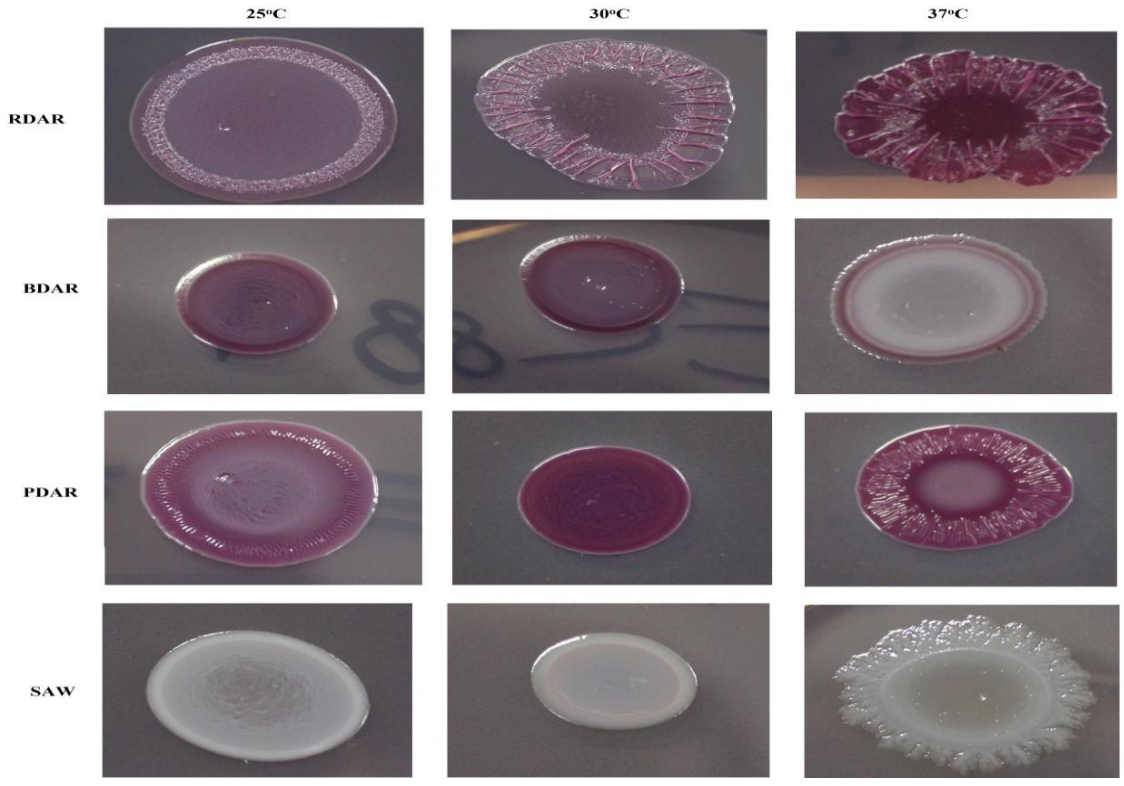


Figure 3-1. Morphotypes expressed by *E. coli* O26 strains on Congo Red Indicator agar at 25, 30 and 37°C. Morphotypes are: RDAR; expresses curli fimbriae and cellulose, BDAR; expresses curli fimbriae, PDAR; express cellulose, SAW; no curli fimbriae or cellulose. Cells were grown on Congo Red Indicator agar plates for 24 hr at 37°C, 48 hr at 30°C or 72 hr at 25°C.

3.5.2 Motility

Evaluating *E. coli* O26 strains for their motility on 0.3% agar revealed that 31 (77.5%) of strains were motile. When 0.2% motility agar was used for nine strains that did not show motility on 0.3% agar, a further three (7.5%) strains ere motile. Lack of motility was a common characteristic in aEPEC strains with six (75%) of them showing non-motile (Table 3.1). Motility was observed in all EHEC strains regardless of source.

Table 3-1. List of *E.coli* O26 strains used in this study, genotypic characteristics, morphotypes, motility and *mlrA* status

Strains	Source	*Stx*[1]	*eae*	*ehx*	*rmal-SNP*	*ecf*	Pathotype	ECM[2] 37/30/25°C	Motility	*mlrA*
EC1A	Cattle	*Stx1*	+	+	+	+	EHEC	SAW/SAW/SAW[3]	M[6]	Occupied
EC1113B	Cattle	*Stx1*	+	+	+	+	EHEC	PDAR/PDAR/PDAR[4]	M	Intact
EC1643B	Cattle	*Stx1*	+	+	+	+	EHEC	PDAR/PDAR/PDAR	M	Intact
EC1857	Cattle	*Stx1*	+	+	+	+	EHEC	PDAR/PDAR/PDAR	M	Intact
EC217	Cattle	*Stx1*	+	+	+	+	EHEC	SAW/SAW/SAW	M	Intact
EC3455	Cattle	*Stx1*	+	+	+	+	EHEC	RDAR[5]/BDAR/BDAR	M	Intact
EC3522	Cattle	*Stx1*	+	+	+	+	EHEC	SAW/SAW/SAW	M	Intact
EC3547A	Cattle	*Stx1*	+	+	+	+	EHEC	PDAR/PDAR/PDAR	M	Intact
EC3652B	Cattle	*Stx1*	+	+	+	+	EHEC	BDAR6/PDAR/PDAR	M	Intact
EC3659B	Cattle	*Stx1*	+	+	+	+	EHEC	BDAR/PDAR/PDAR	M	Intact
EC3671A	Cattle	*Stx1*	+	+	+	+	EHEC	BDAR/PDAR/PDAR	M	Intact
EC3738B	Cattle	*Stx1*	+	+	+	+	EHEC	SAW/SAW/SAW	M	Occupied
EC3743A	Cattle	*Stx1*	+	+	+	+	EHEC	BDAR/PDAR/PDAR	M	Intact
EC4	Cattle	*Stx1*	+	+	+	+	EHEC	SAW/SAW/SAW	M	Occupied
EC478b	Cattle	*Stx1*	+	+	+	+	EHEC	BDAR/PDAR/PDAR	M	Intact
EC674	Cattle	*Stx1*	+	+	+	+	EHEC	BDAR/PDAR/PDAR	M	Intact
EC7B	Cattle	*Stx1*	+	+	+	+	EHEC	BDAR/PDAR/PDAR	M	Intact
EC4158QH1	Clinical	*Stx1*	+	+	+	+	EHEC	SAW/SAW/SAW	M	Occupied
EC4159QH2	Clinical	*Stx1*	+	+	+	+	EHEC	SAW/SAW/SAW	M	Occupied
EC4160QH3	Clinical	*Stx1*	+	+	+	+	EHEC	BDAR/PDAR/PDAR	M	Intact
EC3213QH34	Clinical	*Stx1*	+	+	+	+	EHEC	BDAR/PDAR/PDAR	M	Intact
EC4161QH4	Clinical	*Stx1*	+	+	+	+	EHEC	BDAR/SAW/SAW	M	Occupied
EC4162QH5	Clinical	*Stx1*	+	+	+	+	EHEC	PDAR/PDAR/PDAR	M	Intact
EC4163QH6	Clinical	*Stx1*	+	+	+	+	EHEC	BDAR/PDAR/PDAR	M	Intact
EC4164QH7	Clinical	*Stx1*	+	+	+	+	EHEC	SAW/SAW/SAW	M	Intact
EC4165QH8	Clinical	*Stx1*	+	+	+	+	EHEC	BDAR/SAW/SAW	M	Occupied
EC4166QH9	Clinical	*Stx1*	+	+	+	+	EHEC	PDAR/PDAR/PDAR	M	Intact
EC801	Cattle	-	+	+	+	+	pEHEC	SAW/BDAR/BDAR	M	Intact
EC3983A	Cattle	-	+	+	+	+	pEHEC	BDAR/PDAR/PDAR	M	Intact
EC3989A	Cattle	-	+	+	+	+	pEHEC	PDAR/PDAR/PDAR	M	Intact
EC3435A	Cattle	-	+	-	-	-	aEPEC	SAW/SAW/SAW	NM[7]	Intact
EC3457	Cattle	-	+	-	-	-	aEPEC	SAW/SAW/SAW	M	Intact
EC3610A	Cattle	-	+	-	-	-	aEPEC	SAW/SAW/SAW	NM	Intact
EC3727A	Cattle	-	+	-	-	-	aEPEC	SAW/SAW/SAW	NM	Intact
EC3735A	Cattle	-	+	-	-	-	aEPEC	SAW/SAW/SAW	NM	Intact
EC3768A	Cattle	-	+	-	-	-	aEPEC	SAW/SAW/SAW	NM	Intact
EC4013A	Cattle	-	+	-	-	-	aEPEC	SAW/SAW/SAW	NM	Intact
EC4039A	Cattle	-	+	-	-	-	aEPEC	SAW/SAW/SAW	M	Occupied
EC3536B	Cattle	-	-	-	-	-	NTEC	BDAR/BDAR/BDAR	M	Intact
EC3946A	Cattle	-	-	-	-	-	NTEC	SAW/BDAR/BDAR	M	Intact

[1]All strains were negative for *stx2*: [2]ECM: extracellular matrix components. [3]SAW: no curli fimbriae or cellulose; [4] PDAR: cellulose;

[5]RDAR: curli and cellulose; [6]BDAR: curli; [7]M: Motile; [8]NM: Non motile.

3.5.3 Prophage insertion in *mlrA*

Seven EHEC and a single aEPEC (Table 3.1) displayed a prophage insertion at *mlrA* (Primer F/B=702 bp). Strains that carry a prophage inserted at *mlrA* was found to express the SAW morphotype at 25˚C. The percentage of EHEC from clinical cases with a prophage insertion at *mlrA* was 40% which is higher than that detected in EHEC from cattle (17.6%).

3.5.4 Cell surface hydrophobicity

The mean hydrophobicity values of *E. coli* O26 strains of EHEC, pEHEC, aEPEC and NTEC at 37 and 25˚C are shown in Figure 3.2. The percentage of bound cells to xylene and hexadecane was determined at 25 and 37˚C by BATH assay. Overall, the mean hydrophobicity (%) of strains obtained with xylene was shown to be higher at 37˚C (18.7%) than at 25˚C (1.7%). In contrast, no significant differences were observed in the mean hydrophobicity values of strains using hexadecane at 25˚C (13.2%) or 37˚C (12.7%). Among the four pathotypes, NTEC had significantly higher mean hydrophobicity values than aEPEC, EHEC and pEHEC at 37˚C. When hydrophobicity was determined at 25˚C, NTEC and aEPEC were significantly more hydrophobic than EHEC and pEHEC. Hydrophobicity measurements obtained for human and cattle EHEC strains showed no significant differences at 37 or 25˚C regardless of the hydrocarbon used to determine their cell surface hydrophobicity.

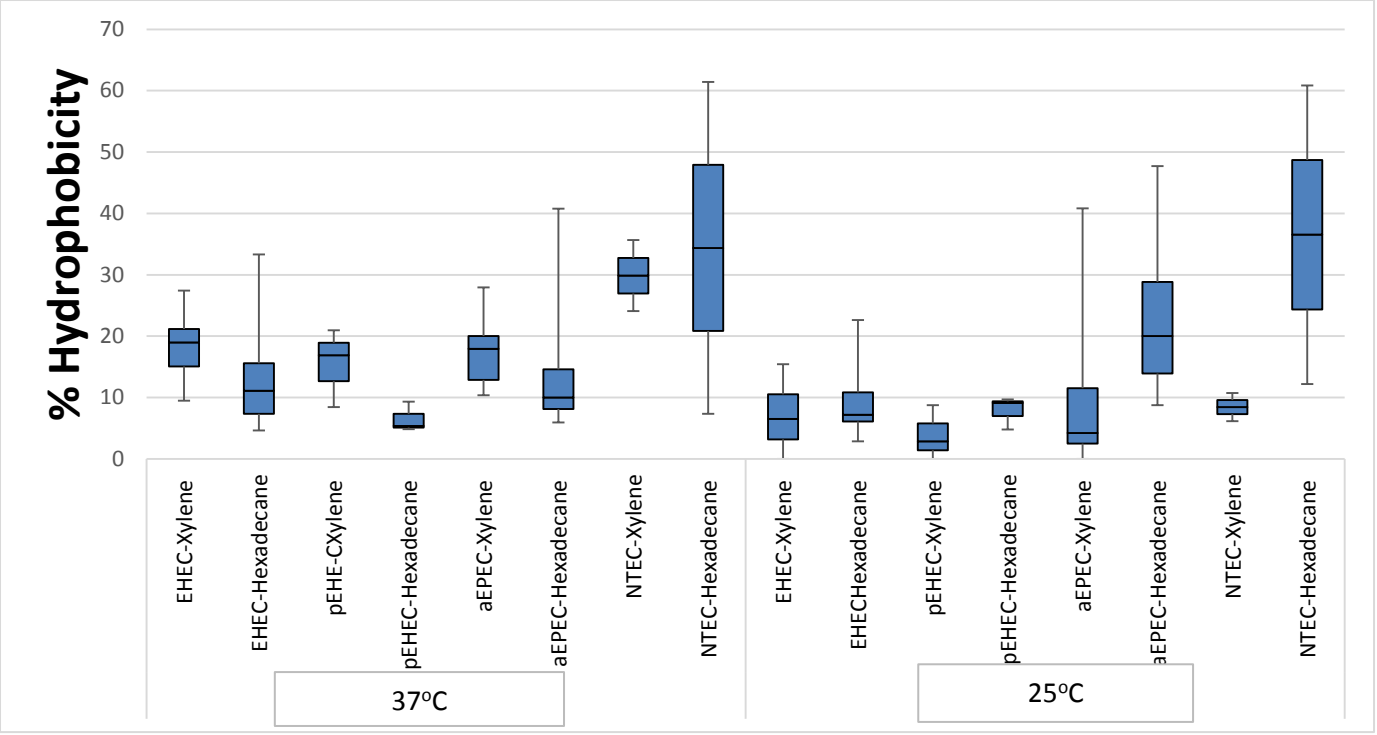


Figure 3-2. Box-plot of cell surface hydrophobicity of *E. coli* O26 pathotypes as measured at 37 and 25°C using xylene and hexadecane. Results represent the average of percentage of bound cells to xylene and hexadecane. Data were grouped by the cell pathotypes. For each box, the lower hinge, upper hinge and inside line represent the 25th (Q1) percentile, the 75th (Q3) percentile and the median, respectively. Lower and upper bars represent the lower and the upper whiskers respectively.

3.5.5 Biofilm formation on polystyrene microtiter plates

Assessment of biofilm formation on polystyrene microtiter plates showed that the ability to form biofilm and the quantity of biofilm produced by the forty *E. coli* O26 strains vary after incubation for 24, 48, or 72 hr. At 24 hr, only four (three EHEC and a single aEPEC) strains showed biofilm production and all four were classified as low producers ($As_{570} \geq 0.08$-$As_{570} \leq 0.16$). At 48 hr, 20 strains displayed biofilm formation with 14 strains classified as low producers ($As_{570} \geq 0.078$-$As_{570} \leq 0.156$), four strains were moderate producers ($As_{570} > 0.156$ - $As_{570} \leq 0.312$) and thick biofilm formation ($As_{570} > 0.312$) was observed with two strains. At 72 hr, 28 strains

displayed capability to produce biofilm on polystyrene plates of which 15 were categorised as low producers ($As_{570} \geq 0.071$-$As_{570} \leq 0.142$), six strains were moderate biofilm ($As_{570} > 0.142$-$As_{570} \leq 0.284$) producers and seven strains developed thick biofilm mass ($As_{570} > 0.284$). EHEC were significantly more likely to produce biofilms at 48 hr in comparison to aEPEC strains, however these differences did not persist at 72 hr. Nonetheless, thick biofilm mass was observed in 33.3% of EHEC in comparison to 12.5% of aEPEC. Considering human and cattle strains, eight (80%) EHEC clinical strains and 14 (82.3%) EHEC cattle strains were able to form biofilm. When comparing the importance of attributes namely ECM components, motility, intact *mlrA* gene in biofilm formation, it was observed that they were significantly more likely to be found in strong and moderate biofilm formers and 48 hr biofilm producers than lower or biofilm-deficient strains, (*P* value <0.001). In addition, the low to limited capacity of biofilm formation at 25˚C appears to be associated with a prophage insertion in *mlrA* or lack of ECM morphotype in EHEC but with lack of motility, expression of SAW morphotype and interrupted *mlrA* in aEPEC (Table 3.1). It was also observed that there was little overlap between impairments in these attributes. For example, SAW morphotype in EHEC non-biofilm forming strains were observed along with interrupted *mlrA* in five strains and SAW morphotype were displayed along with lack of motility in five aEPEC strains. An exception of this was EC4164QH7 which had mutation in RpoS (data not published) and EC4165QH8 which had both mutation in RpoS (data not published) and interrupted *mlrA* but were able to form moderate biofilm mass at 48 hr. Finally, cell surface hydrophobicity had no observed impact on biofilm formation (Figure 3.3).

Table 3-2. Biofilm formation on polystyrene microtiter plates, stainless steel coupons, glass slides and pellicle formation at the air-liquid interface

Pathotypes	Isolates No.	Biofilm mass on polystyrene at incubation time of			Biofilm[5]		Pellicle formation[6]
		24 hr	48 hr	72 hr	SS	GS	
EHEC	EC1A	0.024±0.004[1]	0.076±0.015	0.049±0.007	1	1	0.061±0.011
	EC1113B	0.029±0.005	0.119±0.022*	0.191±0.039**	3	3	**1.884±0.259**
	EC1643B	0.047±0.006	0.251±0.033**[3]	**1.126±0.153**	3	3	**3.595±0.191**
	EC1857	0.036±0.006	0.063±0.011	0.107±0.016*	3	3	0.856±0.078**
	EC217	0.054±0.009	0.100±0.015*	0.043±0.008	2	1	0.025± 0.009
	EC3455	0.066±0.008	0.025±0.005	0.060±0.007	3	3	**1.228±0.145**
	EC3522	0.060±0.008	0.058±0.010	0.086±0.011*	1	1	0.039±0.034
	EC3547A	0.043±0.003	0.236±0.014**	**0.503±0.030**	2	2	0.839±0.113**
	EC3652B	0.056±0.010	0.102±0.014*	0.104±0.016*	3	3	**1.875±0.103**
	EC3659B	0.026±0.009	0.058±0.009	0.093±0.014*	3	3	**1.652±0.152**
	EC3671A	0.016±0.005	0.091±0.010*	0.125±0.017*	3	3	**1.554±0.167**
	EC3738B	-0.010±0.003	0.032±0.013	0.093±0.027*	1	1	0.145±0.030
	EC3743A	0.038±0.006	0.087±0.011*	0.075±0.010*	3	3	**1.218±0.087**
	EC4	0.023±0.004	0.045±0.009	0.024±0.005	2	1	0.097±0.023
	EC478B	0.086±0.011*[2]	0.127±0.011*	0.133±0.011*	2	2	0.480±0.061**
	EC674	0.011±0.005	0.064±0.013	0.130±0.023*	3	3	**1.660±0.151**
	EC7B	0.082±0.014*	0.109±0.013*	0.230±0.052**	3	3	**1.397±0.091**
	EC4158-QH1	0.047±0.005	0.082±0.015*	0.069±0.011	1	1	0.092±0.042
	EC4159-QH2	0.049±0.007	0.089±0.013*	0.055±0.007	2	1	0.035±0.025
	EC4160-QH3	0.098±0.015*	0.034±0.008	0.044±0.008	2	3	**2.257±0.099**
	EC3213-QH34	0.036±0.006	0.072±0.007	0.171±0.017**	3	3	**1.079±0.046**
	EC4161-QH4	0.075±0.012	0.060±0.010	0.061±0.009	2	1	0.050±0.021
	EC4162-QH5	0.001±0.009	0.088±0.020*	**1.021±0.154**	3	3	**2.882±0.255**
	EC4163-QH6	0.017±0.003	0.135±0.030*	**1.469±0.304**	2	3	**1.848±0.127**
	EC4164-QH7	0.053±0.006	0.223±0.025**	0.245±0.025**	2	1	0.092±0.034
	EC4165-QH8	0.070±0.008	0.127±0.019*	0.156±0.026**	1	1	0.065±0.037
	EC4166-QH9	0.061±0.007	0.051±0.010	0.070±0.010	3	3	**2.746±0.163**
pEHEC	EC801	0.040±0.005	0.119±0.017*	0.121±0.020*	2	1	0.150±0.042
	EC3983A	0.033±0.006	0.057±0.011	0.074±0.010*	2	1	0.081±0.024
	EC3989A	0.063±0.008	**0.638±0.036**[4]	**1.402±0.203**	3	3	**3.859±0.138**
aEPEC	EC3435A	0.044±0.009	0.041±0.007	0.060±0.007	0	1	0.104±0.064
	EC3457	0.110±0.020*	0.134±0.023*	0.135±0.014*	1	1	-0.001±0.030
	EC3610A	0.019±0.003	0.017±0.004	0.033±0.005	1	0	0.018±0.023
	EC3727A	0.037±0.005	0.034±0.007	0.119±0.021*	0	1	0.268±0.051
	EC3735A	0.046±0.011	0.017±0.011	0.108±0.019*	0	1	0.135±0.027
	EC3768A	0.035±0.012	0.078±0.017	0.206±0.028**	0	1	0.128±0.020
	EC4013A	0.048±0.018	0.027±0.011	0.101±0.014*	0	1	0.177±0.032
	EC4039A	0.006±0.003	0.060±0.009	0.048±0.007	1	0	-0.005±0.013
NTEC	EC3536B	0.052±0.005	0.224±0.021**	**0.475±0.034**	3	3	**0.966±0.158**
	EC3946A	0.052±0.006	**0.313±0.056**	**0.404±0.034**	3	3	0.587±0.127**
Biofilm category	Low biofilm	>0.08-≤ 0.16	>0.078-≤ 0.156	>0.071-≤ 0.142	1	1	Visual examination of pellicle layer
	Medium biofilm	>0.16-≤0.32	> 0.156-≤ 0.312	>0.142- ≤0.284	2	2	
	Thick biofilm	>0.32	> 0.312	> 0.284	3	3	
Negative control	LB broth	0.080±0.0009	0.078±0.001	0.071±0.0004	0	0	

[1]Values are shown as mean of biofilm production ± standard error on polystyrene surfaces, According to the biofilm mass quantified with crystal violet staining assay at 570 nm, strains were labelled as [2]*low biofilm, [3] **medium biofilm, [4]**thick biofilm**. [5]Biofilm formation on stainless steel (SS) and glass slides (GS) was scored visually; and given a score from 0 (No biofilm observed) to 3 (thick biofilm). [6]The presence or absence of visible pellicle biofilms was scored visually before staining with CV.

3.5.6 Biofilm formation on stainless steel coupons and glass slides

The results of biofilm formation on stainless steel coupons and glass slides by *E. coli* O26 strains in this study are shown in Table 3.2. The majority of the strains had the capacity to form biofilms on the surface of stainless steel coupons (87.5%) and glass slides (95%) at the air-liquid interface. In terms of pathotypes, 27 EHEC (100%), three pEHEC (100%) and two NTEC (100%) were significantly more likely to produce biofilms on stainless steel at the air-liquid interface at 72 hr in comparison to three aEPEC strains (37.5%). However, these differences did not persist when glass slides were used to investigate biofilm formation with six aEPEC (75%) forming a biofilm on glass slides. Comparative analysis of biofilm formation by human and cattle EHEC did not identify differences in biofilm formation between sources. Under the conditions used in this study, strains with the following profiles: motile, ECM components (curli and or cellulose), and intact *mlrA* showed thicker biofilm mass at the air-liquid interface (score 2 or 3) on stainless steel and glass slides than strains that lacked these profiles; (*P* value <0.001). The possible influence of hydrophobicity on biofilm formation was also investigated; however, no correlation was found (Figure 3.3).

3.5.7 Pellicle formation at the air-liquid interface

The presence of visible pellicle biofilms at the air-liquid interface was scored visually before staining with CV. When strains were tested for their capacity to form a pellicle layer attached to the wall of a glass tube at the air-liquid interface, 20 of 40 strains (50%) displayed pellicle formation at the air-liquid interface. At the pathotype level, 17 EHEC ranked moderate to high pellicle producers in comparison to aEPEC which did not form a biofilm layer at the air-liquid

interface. A single pEHEC strain and both NTEC strains formed thick pellicles. In addition, a significant correlation was observed between thick biofilm producers on polystyrene, stainless steel and glass slides and pellicle formation. However, pellicle formation was not an indicator for biofilm formation on those surfaces. O26 strains capable of producing well attached pellicle at the air-liquid interface were motile and expressed curli or cellulose and had intact *mlrA* as opposed to strains that did not exhibit these characteristics (*P* value <0.001). Finally, no correlation was found between cell surface hydrophobicity and pellicle formation (Figure 3.3).

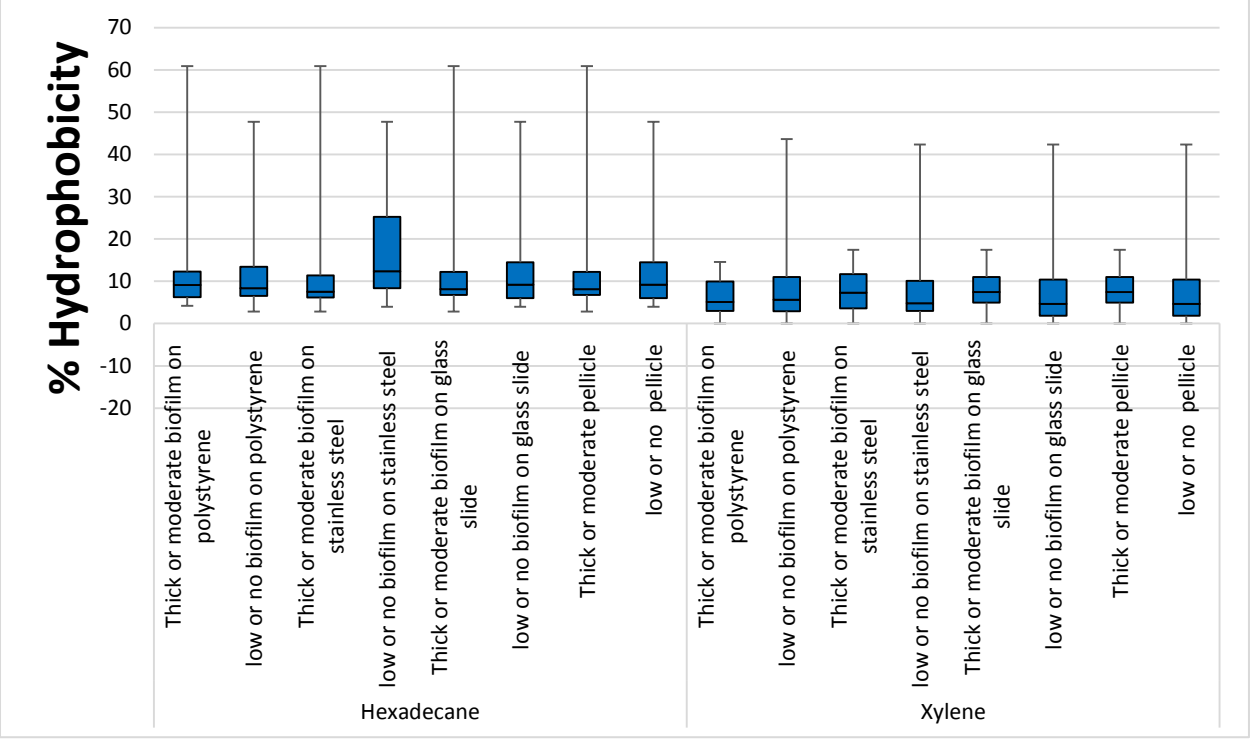


Figure 3-3. Box-plot of cell surface hydrophobicity of *E. coli* O26 strains as measured at 25°C using hexadecane and xylene and its influence on biofilm formation. Results represent the average of percentage of bound cells to hexadecane and xylene. Data were grouped by the capacity of biofilm formation on polystyrene microtiter plates, stainless steel coupons and glass slides pellicle at the air-liquid interface. For each box, the lower hinge, upper hinge and inside line represent the 25th (Q1) percentile, the 75th (Q3) percentile and the median, respectively. Lower and upper bars represent the lower and the upper whiskers respectively.

3.5.8 Tolerance of *E.coli* O26 biofilm cells to disinfectants

The influence of disinfectants on *E. coli* O26 cell viability within the biofilms was determined by enumerating viable cells remaining after 0.45% Kwiksan 22 (QAC), 1% Profoam (QAC) and 1% Topactive Des. treatment (Table 3.3). After 2 min exposure, none of the disinfectants were able to completely inactivate all cells within a biofilm. Exposure to Topactive Des. resulted in 0.03 to 0.76 $\log_{10}$ reduction. Treatment with Profoam had a greater effect on biofilm cells, led to 0.02-1.74 $\log_{10}$ reductions. When strains were exposed for 2 min to Kwiksan 22 (QAC) biofilm cells reduction ranged from 0.05- 1.77 $\log_{10}$ CFU per well. In terms of pathotypes, Kwiksan 22 has shown to be the most effective sanitiser against all pathotypes whereas Topactive Des. was the least effective.

Table 3-3. Exposure of *E. coli* O26 biofilms to Topactive Des., Kwiksan 22 and Profoam disinfectants for 2 min

Pathotypes	Strains	2 min exposure to sanitization treatment					
		PBS	Topactive Des.	PBS	Kwiksan	PBS	Profoam
EHEC	EC1113b	7.09 (±0.1)	6.94 (±0.2)	6.77 (±0.2)	6.71(±0.02)	7.31(±0.05)	5.56 (±0.8)
	EC1643b	6.64 (±0.5)	6.45 (±0.4)	7.04 (±0.1)	5.47(±0.6)	6.99(±0.3)	6.11 (±0.2)
	EC1857	7 (±0.2)	6.74 (±0.3)	6.60 (±0.4)	6.25(±0.5)	6.58(±0.4)	6.58 (±0.5)
	EC217	6.9 (±0.2)	6.91 (±0.2)	6.58 (±0.2)	5.34(±0.5)	6.29(±0.3)	5.77 (±0.5)
	EC3522	6.08 (±0.2)	6.05 (±0.5)	6.22 (±0.4)	5.46(±0.5)	6.18(±0.3)	5.53 (±0.3)
	EC3547A	6.47 (±0.2)	6.42 (±0.3)	6.58 (±0.3)	5.50(±0.8)	6.29(±0.6)	6.27 (±0.5)
	EC3652B	6.60 (±0.4)	6.4 (±0.4)	6.55 (±0.5)	5.36(±0.7)	6.30(±0.4)	5.67 (±0.4)
	EC3659B	6.65 (±0.5)	6.40 (±0.6)	6.25 (±0.2)	5.04(±0.1)	6.21(±0.9)	4.53 (±1.0)
	EC3671A	6.65 (±0.2)	6.17 (±0.1)	7.11 (±0.3)	6.26(±1.0)	6.84(±0.1)	5.85 (±0.7)
	EC3738B	7.35 (±0.07)	6.97 (±0.1)	6.79 (±0.2)	5.27(±0.2)	6.37(±0.1)	5.25 (±0.3)
	EC3743A	7.25 (±0.2)	7.25 (±0.2)	6.53 (±0.4)	5.84(±0.8)	6.72(±0.7)	5.93 (±1.15)
	EC478b	6.68 (±0.4)	6.52 (±0.3)	7.15 (±0.05)	5.70(±0.6)	6.67 (±0.4)	5.14 (±0.9)
	EC674	6.45 (±0.8)	6.24 (±0.9)	6.45 (±0.5)	6.14 (±0.8)	6.70 (±0.5)	6.66 (±0.5)
	EC7B	6.95 (±0.3)	6.46 (±0.2)	6.48 (±0.6)	5.50 (±0.01)	6.77 (±0.2)	5.71 (±0.3)
	EC4158QH1	7.10(±0.1)	7.01 (±0.04)	6.45 (±0.3)	5.92(±0.4)	6.51(±0.4)	5.96 (±0.6)
	EC4159QH2	6.67 (±0.2)	6.33 (±0.3)	6.94 (±0.2)	5.59(±0.5)	6.71(±0.1)	6.19 (±0.02)
	EC4160QH3	6.20 (±0.3)	5.81 (±0.7)	6.00 (±0.3)	6.00 (±0.3)	5.96 (±0.1)	5.72 (±0.5)
	EC3213QH34	6.61 (±0.1)	6.43 (±0.4)	6.82 (±0.3)	6.75(±0.1)	6.71(±0.5)	6.22 (±0.5)
	EC4162QH5	6.76(±0.1)	6.62 (±0.2)	6.12 (±0.6)	5.58 (±0.9)	6.76 (±0.2)	6.30 (±0.3)
	EC4163QH6	6.88 (±0.4)	6.57 (±0.4)	6.42 (±0.5)	6.14 (±0.5)	6.65 (±0.5)	5.90 (±0.7)
	EC4164QH7	5.01 (±0.5)	4.25 (±0.1)	5.56 (±0.9)	5.19 (±0.8)	6.21 (±0.4)	5.31 (±0.1)
	EC4165QH8	6.00 (±1.0)	6.00 (±1.0)	5.89 (±0.6)	4.82 (±1.1)	6.93 (±0.2)	6.05 (±0.7)
pEHEC	EC801	6.4 (±0.3)	5.95 (±0.8)	6.19 (±0.3)	5.04 (±0.7)	6.31 (±0.5)	5.11 (±0.9)
	EC3983A	6.74 (±0.5)	6.37 (±0.6)	6.38 (±0.2)	4.95(±0.6)	6.44(±1.0)	5.60 (±0.9)
	EC3989A	7.16 (±0.1)	7.16 (±0.074)	7.35 (±0.10)	6.21(±0.11)	7.36(±0.07)	6.13 (±0.05)
aEPEC	EC3457	6.25 (±0.3)	5.68 (±0.390)	6.48 (±0.46)	4.71(±1.25)	6.47(±0.50)	4.93 (±1.4)
	EC3727A	6.30 (±0.09)	6.27 (±0.044)	6.18 (±0.41)	5.45(±0.32)	6.42(±0.26)	5.52 (±0.04)
	EC3735A	6.46 (±0.2)	6.28 (±0.386)	6.76 (±0.28)	5.98(±0.32)	6.42(±0.22)	5.72 (±0.4)
	EC3768A	6.09 (±0.2)	5.84 (±0.050)	6.20 (±0.95)	5.87(±1.32)	6.31(±0.40)	6.03 (±0.6)
	EC4013A	6.49 (±0.3)	6.14 (±0.173)	5.99 (±0.18)	5.49(±0.73)	6.24(±0.69)	5.89 (±0.1)
NTEC	EC3536B	6.81 (±0.6)	6.61 (±0.443)	6.48 (±0.60)	5.65(±1.00)	6.52(±0.39)	6.37 (±0.3)
	EC3946A	7.33 (±0.1)	7.01 (±0.155)	7.05 (±0.05)	7.00(±0.00)	7.23(±0.02)	6.75 (±0.1)

Biofilms were formed in polystyrene plates and data are shown as mean $\log_{10}$ CFU per well (±the standard errors of the means). The influence of disinfectants on biofilms was determined by enumerating viable cells remaining after treatment with 1% Topactive Des., 0.45% Kwiksan 22 (QAC) and 1% Profoam (QAC) treatment and compared to that of PBS control. Results are averaged from at least 3 biological replicates.

Among pathotypes, the mean reduction caused by Kwiksan was greatest in pEHEC (1.145 $\log_{10}$ CFU/well) and lowest in those of NTEC (0.44 $\log_{10}$ CFU/well). Profoam resulted in the same mean $\log_{10}$ CFU/well reduction for both EHEC (0.70 $\log_{10}$ CFU/well) and aEPEC (0.70 $\log_{10}$ CFU/well) but had a greater mean reduction level in pEHEC (1.19 $\log_{10}$ CFU/well) and NTEC (0.32 $\log_{10}$ CFU/well). In contrast, when biofilm formed by all pathotypes were treated with Topactive Des. the mean of viable cell counts was reduced by 0.2 to 0.3 $\log_{10}$ CFU/well. Statistical analysis of means indicated that pathotypes, biofilm density, production of one or both

of the extracellular components had no impact on *E. coli* O26 biofilm cells survival to disinfectants treatment. Clinical and cattle strains showed various level of tolerance to disinfectant with cattle strains were being more susceptible to disinfectant intervention than their human counterparts (Figure 3.4) although not statistically significant.

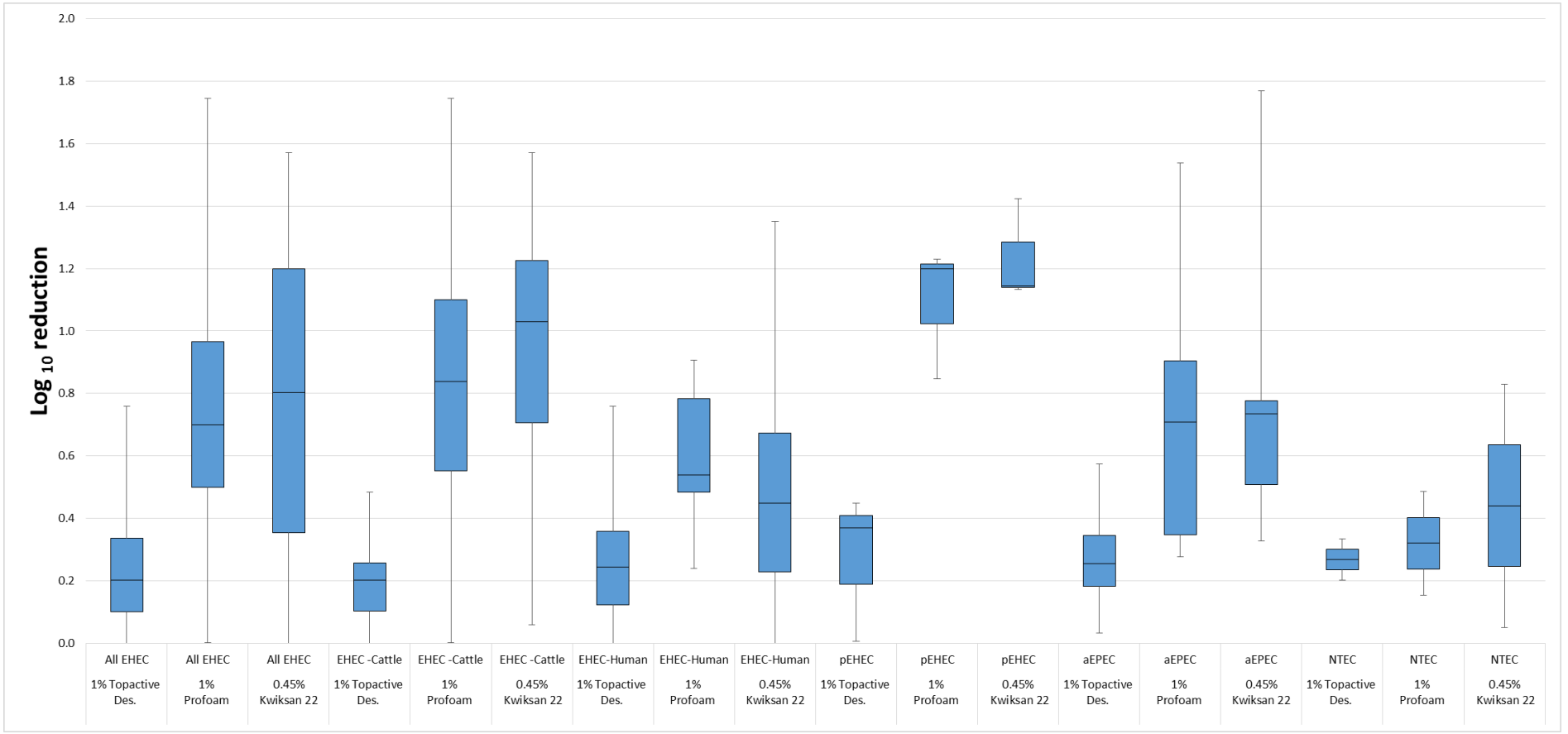


Figure 3-4. Box-plot of log_{10} reduction of *E. coli* O26 after 2 min exposure to Topactive Des., Profoam and Kwiksan 22 based on strains source and pathotypes. For each box, the lower hinge, upper hinge and inside line represent the 25th (Q1) percentile, the 75th (Q3) percentile and the median, respectively. Lower and upper bars represent the lower and the upper whiskers respectively

3.6 **Discussion**

EHEC of serogroup O26 have been associated with foodborne disease outbreaks worldwide (Ethelberg et al., 2009, EFSA. and ECDC, 2014). Formation and encasement of *E. coli* O26 cells in a complex biofilm matrix may enhance resistance to antimicrobials agents under various conditions (Reviewed in (Srey et al., 2013)). There are a number of described methods for biofilm assessment on abiotic surfaces. However, no standard accepted biofilm methodology has been published to date. In this study we choose to utilize the low salt Lb for a number of reasons. Increase in the number of adherent cells was seen with E. coli strains in nutrient-rich medium such as low salt LB or without salt LB or tryptic soy broth (TSB) while low capacity of low biofilm formation was observed in nutrient-defined medium such as (M9) and diluted meat juice (DMJ) (Hood and Zottola, 1997, Bokranz et al., 2005). In addition, Bokranz et al. (2005) observed that biofilm formation in LB medium without salt correlated with the colony morphotype on CR agar plates. The use of LB media and protocols previously utilised to study biofilm phenotypes in *E. coli* will facilitate comparison with other studies when possible.

The current study investigated whether biofilm production is associated with particular pathotypes, curli and/or cellulose production, motility, intact *mlrA*, and hydrophobicity. Results presented here demonstrated that a prophage insertion in *mlrA*, lack of motility and failure in producing ECM prevented or lowered biofilm formation with overlapping between these attributes was observed. In EHEC, curli expression was observed more frequently at 37˚C but cellulose expression was the most predominant morphotype at 30 and 25˚C. In addition, strains produced cellulose or curli at 25˚C were able to produce biofilm on at least one of the tested surfaces (Table 3.2). This is in agreement with a previous finding of Uhlich

et al., (2014) who found that in EHEC non-O157, cellulose is suppressed at 37˚C but produced at 30˚C and 25˚C and that production of cellulose or curli or both were associated with biofilm production. In aEPEC strains, SAW morphotype was the predominant characteristic at all tested temperatures and low biofilm formation was observed, in contrast; BDAR was displayed almost at all temperatures in NTEC (Table 3.1) and strong biofilm formation was observed. The obtained result could be due to the fact that both curli (BDAR) and cellulose (PDAR) production are dependent on the expression of *csgD* which is influenced by temperature (the only variable tested here), pH and available nutrients (Ogasawara et al., 2010). In addition, the insertion of a prophage in *mlrA* in eight strains (Table 3.1) and mutation in RpoS in only two strains (EC4164QH7 and EC4165QH8; supplementary information) could explain the production of SAW morphotype by a number of strains. Finally, lack of motility was observed only in aEPEC (75%) and could be another attribute that limit ECM expression and biofilm formation. It has been reported that lack of motility could inhibit biofilm formation by preventing curli expression in bacteria that could not be complemented for curli by restoring *mlrA* (Chen et al., 2013). However, the remaining strains (Table 3.1) that were motile with intact *mlrA* and wild type RpoS (all strains had wild type RpoS except EC4164QH7 and EC4165QH8; data not published) but exhibited SAW morphotype could have additional, yet to be discovered structural or regulatory gene mutations.

The influence of physiochemical properties represented by cell surface hydrophobicity on biofilm formation using BATH assay was also investigated. The bacterial adhesion to xylene and hexadecane has been extensively used for measuring cell surface hydrophobicity (Bokranz et al., 2005, Nesse et al., 2014, Goulter et al., 2010, Rivas et al., 2005). Comparing cell surface hydrophobicity of strains by measuring adherence to hydrocarbons showed

differences between strains in their affinity to the two chemicals. Differences in the degree of adherence to xylene and hexadecane have been previously observed (Rivas et al., 2005). It was speculated that each hydrocarbon might measure different aspects of hydrophobicity (Rivas et al., 2005, Goulter et al., 2010). Among the four pathotypes tested in this study, NTEC strains had significantly higher hydrophobicity values than other pathotypes at all tested temperatures. The high hydrophobicity values for *eae*-negative strains is in agreement with others (Nesse et al., 2014, Rivas et al., 2005) who reported the same observation for *eae*-negative strains of O103:H2 serotype and O157:HR (Rivas et al., 2005). In addition, no correlation was found between hydrophobicity and biofilm formation on any of the surfaces tested or at the air-liquid interface (Figure 3.3), which is consistent with previous studies (Chia et al., 2009, Rivas et al., 2005, Li and McLandsborough, 1999). Together with the results of this study, this suggests that the adhesion process is likely to involve a variety of physicochemical and/or biological factors (Goulter et al., 2010, Pembrey et al., 1999).

The capacity of biofilm formation in *E. coli* O26 strains which were *stx*$^{+}$ (EHEC) and *stx*$^{-}$ (pEHEC, NTEC and aEPEC), with various ECM, *mlrA* and motility profiles on polystyrene plates at 24, 48 and 72 hours and on stainless steel and glass slide was also investigated in this study. The results are in accordance with previous studies of Uhlich et al. (2013) and Chen et al. (2013) who observed a positive role for curli and/or cellulose, motility and intact *mlrA* in biofilm formation. In this study, biofilm development for motile strains with intact *mlrA* and expressing cellulose or curli occurred earlier than other strains regardless of pathotypes on polystyrene surface and developed thick biofilm mass on stainless steel or glass slides, suggesting that the presence of these characteristics may enhance biofilm formation. However, further studies using knockout mutants are required to confirm the role of the abovementioned factors on biofilm formation.

Association between ECM production, motility, intact *mlrA* and pellicle formation at the air-liquid interface was observed in this study. This is in agreement with the observation of Wang et al. (2012) who has shown that the curli-positive strains of serotype O26:H11 exhibited an overall high potency of pellicle formation. Strains that exhibited the SAW phenotype were limited in their capacity to form pellicle at the air-liquid interface in this study. These findings correlate with previous reports (Uhlich et al., 2006) and suggest that ECM expression and biofilm formation by strains with SAW morphotypes are more inducible upon exposure to solid surfaces than at the air-liquid interface (Uhlich et al., 2006).

Moreover, biofilm production on one surface may not correlate with biofilm formation on a different surface. For example, biofilm formation of *E. coli* O26 strains on glass slides at the air- liquid interface (95%) was significantly higher than that on polystyrene plates (70%) and pellicle formation at the air-liquid interface (50%). This finding is in agreement with previous studies where some strains of non-O157 that formed biofilm on one surface were not able to develop biofilm on other surfaces (Nesse et al., 2014, Goulter et al., 2010, Rivas et al., 2007) and suggests that cell contact surfaces can influence biofilm formation. As abiotic surfaces are commonly used in the food industry it may be necessary to evaluate specific surfaces for their capacity to act as a matrix for biofilm formation under conditions relevant to the food and meat industry settings. Furthermore, biofilm formation on various surfaces seems to be a common characteristic not only for EHEC, but also for pEHEC and NTEC pathotypes. It is well documented that *stx*-negative pathotypes can become EHEC via acquisition of *stx* (Toth et al., 2003, Schmidt et al., 1999a, Bielaszewska et al., 2007). Given the nature of food and meat industry surfaces, where bacteria live in multispecies biofilm community, biofilms can act as an environment for dissemination of *stx* and emergence of new pathogenic strains (Nesse et al., 2014, Habimana et al., 2010, Burmolle et al., 2006). Together with the results of

this study, biofilm formation by *stx*-negative strains warrants additional investigation to determine the clinical significance of biofilm formation by this group. Finally, both clinical and cattle strains of EHEC pathotype were able to form biofilm which may suggest that cattle strains represent a source of biofilm-forming bacteria that might occupy food contact surfaces, although additional factors that represent the food and meat processing environments should be considered (Marouani-Gadri et al., 2009, Burmolle et al., 2006, Habimana et al., 2010, Helke et al., 1993, Hood and Zottola, 1997).

EHEC O26 can cause illness range from diarrhoea to severe sequelae such as HUS; therefore interventions to control this pathogen and prevent future outbreaks of illness are required. When *E. coli* O26 strains were challenged to determine the impact of biofilm formation on sensitivity toward the tested disinfectants, a protective effect of biofilm was observed. Interestingly, strains that showed lowest biofilm formation on polystyrene plate were equally resistant to disinfectant intervention as the strong biofilm forming strains (Table 3.2). This is consistent with the study of Vogeleer et al. (2015) where the amount of biofilm mass and expression of cellulose or curli had no impact on the ability of biofilm cells to survive disinfectants treatments. In contrast to others (Park and Chen, 2015, Uhlich et al., 2006, Wang et al., 2012) that suggested curli and/or cellulose appeared to play a critical role in EHEC tolerance to disinfectants. Variations between studies could be attributed to the differences in experimental designs and/or the use of different bacterial strains. Additionally, differences in the response to disinfectants were observed between strains within the same pathotypes in this Chapter, with previous studies also reporting variation in tolerances amongst *E. coli* serogroups including *E. coli* O26 (Uhlich et al., 2006, Wang et al., 2012, Vogeleer et al., 2015). Earlier, in Chapter 2 of this study, we showed that clinical and cattle *E. coli* O26 planktonic cells could not survive the challenge with QAC and peracetic acid

based disinfectants approved for use in Australian food industry at their recommended concentration, regardless of pathotypes. In this Chapter, the majority of isolates did not form biofilm after 24 hr of incubation. Taking these findings together suggests that regular and proper sanitization could be effective to prevent the formation of biofilms in food production environments. However, it is also indicated that other factors such as the pre-conditioning of the substratum, to which the bacteria would attach could increase or inhabit the attachment and subsequently biofilm formation. (Marouani-Gadri et al., 2009, Hood and Zottola, 1997, Helke et al., 1993, Habimana et al., 2010, Burmolle et al., 2006). For example, pre-exposure of the food surfaces to beef juice extract provides a protective matrix for the bacterial cells impeded in (Noyce et al., 2006). In addition, the co-existence with other resistant species in a biofilm would mean that expression of resistance by a species within mixed-biofilm community could provide resistance to the whole community (Burmolle et al., 2006). Furthermore, integration into a biofilm matrix could enhance the opportunities for pathogens that are non-biofilm formers and metabolically inactive cells to survive in meat processing environments (Habimana et al., 2010, Stoodley et al., 2001, Fux et al., 2004).

3.7 **Conclusion**

The study provided insight into the biofilm characteristics of EHEC that caused human infections and those from cattle origin, and other pathotypes. Some factors that appear to enhance or limit biofilm formation in *stx*-positive or *stx*-negative *E. coli* O26 pathotypes have been also investigated. Pathotypes of *E. coli* O26 showed varying capacities to form biofilms, however, most EHEC strains had the capacity to form biofilm on all surfaces and at the air-liquid interface under the conditions used in this study. The ability of biofilm formation provided a protective effect to *E. coli* O26 strains against the three sanitisers, previously

shown to successfully control the growth of their planktonic counterparts. While there are caveats to the results observed in this study, the utility of this study is to provide initial insights into factors that could possibly influence biofilm formation by *E. coli* O26 and then the effect of this phenotype on tolerance to disinfectants. Further studies that represent the food and meat processing environments by considering the effect of co-existence with other microorganisms, presence of organic residues on food surfaces and resistance or adaptation to disinfection are required.

Supplementary information to Chapter 3

Data presented here is part of Chapter 3 but not presented within the prepared manuscript

3.8 Materials and Methods

3.8.1 Prevalence of autotransporter and fimbriae-encoding genes.

The availability of the whole genome sequence of strains involved in this study prompted the screening of the genome for autotransporter and fimbriae encoding genes. *In silico* PCR was performed using fast PCR software to investigate whether the presence of a specific autotransporter (*ehaA "α* and *β"*, *ehaB "α* and *β"*, *ehaC "α* and *β"*, *ehaD"α* and *β"*(Wells et al., 2008) and *ehaG "α"* (Vogeleer et al., 2015) and *"β"* (Totsika et al., 2012), *saa* (Paton et al., 2001) and *yejO* (Vogeleer et al., 2015), fimbriae encoding genes (*loc1* (Vogeleer et al., 2015), *loc2* (Vogeleer et al., 2015), *loc2b* (Vogeleer et al., 2015), *loc3, loc4, loc5, loc6, loc7, loc8, loc9, loc10, loc11, loc12, loc13, loc14*" (Low et al., 2006) was associated with certain phenotypes or biofilm formation.

3.8.2 Mutations in RpoS

The *rpoS* sequence of *E. coli* O26 strains was identified in the assemblies using *in silico* PCR. The *rpoS* sequence then was extracted and translated into protein sequence (RpoS). The translated protein sequence of RpoS from *E. coli* O26 strains were compared to the wild type O157:H7 strain (ATCC 43895) by multiple sequence alignments using MUSCLE (Edgar, 2004).

3.8.3 Tolerance of *E. coli* O26 biofilms to disinfectants

As per section 3.4.8 in Chapter 3 with all strains treated with disinfectants for 10 min.

3.9 **Results**

3.9.1 Prevalence of autotransporter and fimbriae-encoding genes

The prevalence of biofilm associated autotransporter and fimbriae-encoding genes are shown in Figure 3.5. A total of 29 genes were investigated for their presence using *in silico* PCR. All strains were positive for *ehaBβ, ehaCβ, yejO, loc7*, *loc8*, *loc9*, *loc10, loc11* and *z5913* whereas all of them were negative for *ehaBα*, ehaCα, *ehaDβ*, *ehaGβ, saa, loc1 (z0024*), *loc2* (*z0152*), *loc6* (z1538). Other notable findings include, *espP* being present in 96.3% of EHEC and 100% pEHEC but absent in aEPEC and NTEC. *Loc4*, *loc12* and *loc13* were absent in NTEC but was present in all aEPEC, all pEHEC and all but one EHEC strain. Results presented in the current study did not identify any association between the presence of these genes and the ability to form biofilms.

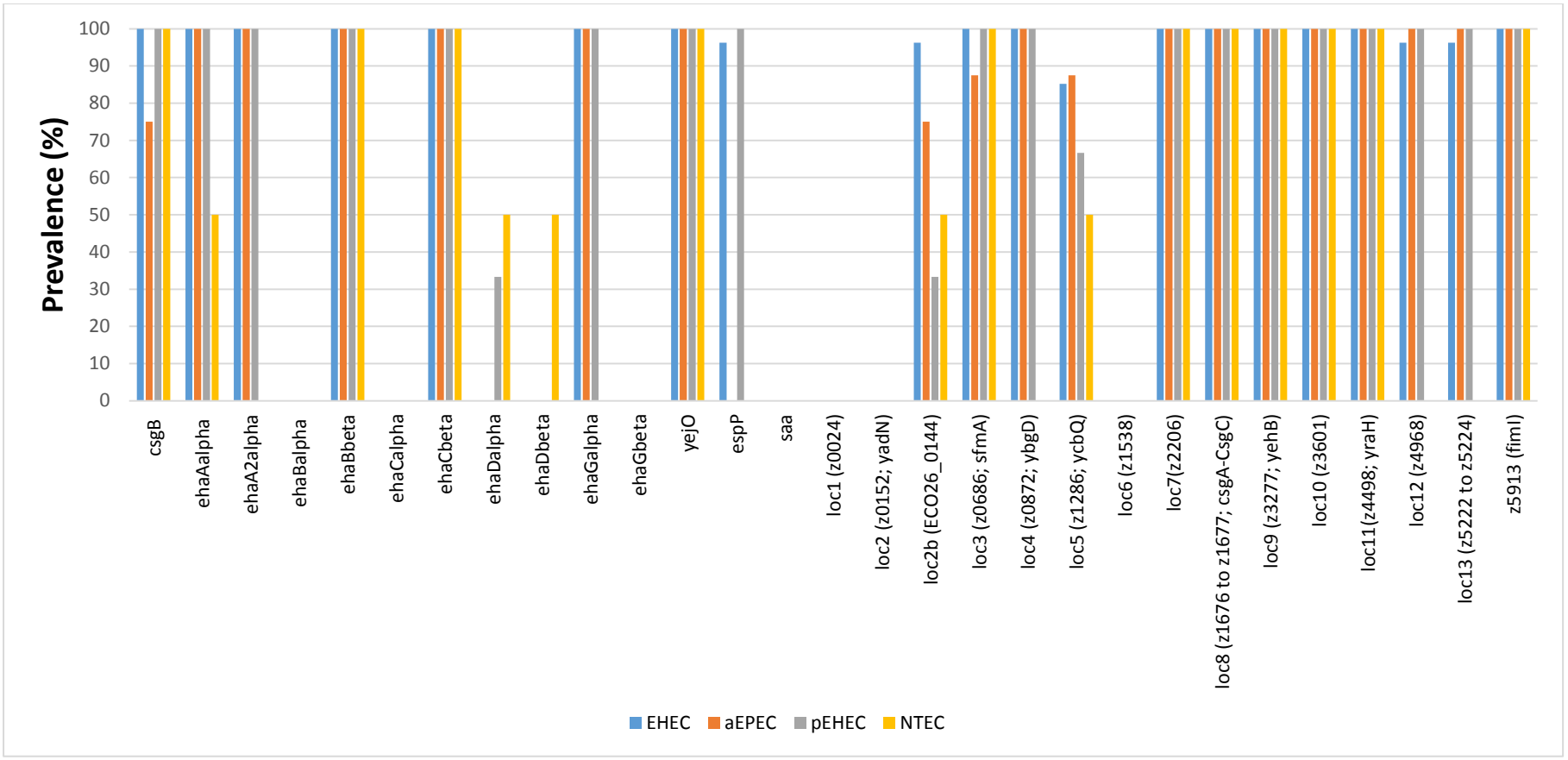


Figure 3-5. Distribution of autotransporter and fimbriae encoding genes among *E. coli* O26 pathotypes. *In silico* PCR was used to identify the presence or absence of the target gene.α amplified the variable sequence of the gene and β: amplified the conserved sequence.

3.9.2 Mutation in RpoS

Alignment of translated protein sequence of RpoS from *E. coli* O26 strains to the wild type O157:H7 strain (ATCC 43895) indicated that mutation in RpoS is not common in *E. coli* O26 strains as 95% had wild type RpoS. RpoS mutations occurred in two EHEC strains (7%) and both were of clinical origin. An EHEC strain (EC4164QH7) showed a single amino acid change (substitution of N to Y) and EC4165QH8 had a single amino acid deletion (Figure 3.6). EC4164QH7 and EC4165QH8 were motile, with SAW morphotype at 25^{o}C and one of them (EC4165QH8) had a prophage inserted in the *mlrA.* These strains were limited in their capacity to form biofilm on glass slides and pellicle at the air-liquid interface but formed moderate biofilm on polystyrene plate (Table 3.2).

```
43895 WT        MSQNTLKVHDLNEDAEFDENGVEVFDEKALVEEEPSDNDLAEEELLSQGATQRVLDATQL
EC4164QH7       MSQNTLKVHDLNEDAEFDENGVEVFDEKALVEEEPSDNDLAEEELLSQGATQRVLDATQL
EC4165QH8       MSQNTLKVHDLNEDAEFDENGVEVFDEKALVEEEPSDNDLAEEELLSQGATQRVLDATQL
                ************************************************************

43895 WT        YLGEIGYSPLLTAEEEVYFARRALRGDVASRRRMIESNLRLVVKIARRYGNRGLALLDLI
EC4164QH7       YLGEIGYSPLLTAEEEVYFARRALRGDVASRRRMIESNLRLVVKIARRYGNRGLALLDLI
EC4165QH8       YLGEIGY-PLLTAEEEVYFARRALRGDVASRRRMIESNLRLVVKIARRYGNRGLALLDLI
                ******* ************************************************

43895 WT        EEGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKELNVYLR
EC4164QH7       EEGYLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKELNVYLR
EC4165QH8       EEGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKELNVYLR
                *** ********************************************************

43895 WT        TARELSHKLDHEPSAEEIAEQLDKPVDDVSRMLRLNERITSVDTPLGGDSEKALLDILAD
EC4164QH7       TARELSHKLDHEPSAEEIAEQLDKPVDDVSRMLRLNERITSVDTPLGGDSEKALLDILAD
EC4165QH8       TARELSHKLDHEPSAEEIAEQLDKPVDDVSRMLRLNERITSVDTPLGGDSEKALLDILAD
                ************************************************************

43895 WT        EKENGPEDTTQDDDMKQSIVKWLFELNAKQREVLARRFGLLGYEAATLEDVGREIGLTRE
EC4164QH7       EKENGPEDTTQDDDMKQSIVKWLFELNAKQREVLARRFGLLGYEAATLEDVGREIGLTRE
EC4165QH8       EKENGPEDTTQDDDMKQSIVKWLFELNAKQREVLARRFGLLGYEAATLEDVGREIGLTRE
                ************************************************************

43895 WT        RVRQIQVEGLRRLREILQTQGLNIEALFRE
EC4164QH7       RVRQIQVEGLRRLREILQTQGLNIEALFRE
EC4165QH8       RVRQIQVEGLRRLREILQTQGLNIEALFRE
                ******************************
```

Figure 3-6. MUSCLE alignment of translated RpoS protein sequence. RpoS mutations occurred in EC4165QH8 by deletion of amino acid number 68 (S) and by substitution of amino acid number 124 (N to Y) in EC4164QH7.

3.9.3 Tolerance of *E. coli* O26 biofilms to disinfectants

To determine the influence of disinfectants on *E. coli* O26 biofilms, after 10 min exposure to sanitisers, viable cell count was performed on both PBS control and disinfectants treated cells. Despite that strains showed variation in their response to disinfectants, none of the disinfectants were able to completely eliminate cells within a biofilm. Viable cell counts of PBS treated cells for the majority of strains fluctuated between ≈5 and 7 $\log_{10}$ CFU per well (Table 3.4). After 10 min exposure the range of reduction ($\log_{10}$ CFU per well) caused by 1% Topactive Des. was 0.015 to 0.47 $\log_{10}$ CFU/well, 1% Profoam resulted in 0.002 to 1.90 $\log_{10}$ CFU/well reduction and 0.45% Kwiksan caused reductions ranging from 0.01 to 1.65 $\log_{10}$ CFU per well.

The effect of disinfectants on *E. coli* O26 pathotypes after 10 min is shown in Figure 3.7. Kwiksan was the most effective disinfectant, resulting in a mean $\log_{10}$ reduction of 0.57 for EHEC (with only four EHEC reduced by >1 $\log_{10}$ CFU/well), 0.51 $\log_{10}$ CFU/well for pEHEC, 0.73 $\log_{10}$ CFU/well for aEPEC (with only one aEPEC reduced by >1 $\log_{10}$ CFU/well) and 0.38 $\log_{10}$ CFU/well for NTEC. Profoam resulted in lower $\log_{10}$ CFU/well reduction than Kwiksan causing 0.36 $\log_{10}$ CFU/well, 0.25 $\log_{10}$ CFU/well, 0.41 $\log_{10}$ CFU/well and 0.15 $\log_{10}$ CFU/well mean $\log_{10}$ reduction of EHEC, pEHEC, aEPEC and NTEC respectively with only a single EHEC strain and a single aEPEC strain reduced by >1 $\log_{10}$ CFU/well). In contrast, Topactive Des. resulted in the lowest mean $\log_{10}$ reduction for all pathotypes with 0.07 $\log_{10}$ reduction of EHEC, 0.12 $\log_{10}$ CFU/well for pEHEC, 0.13 $\log_{10}$ CFU/well for aEPEC and 0.3 $\log_{10}$ CFU/well for NTEC. The maximum reduction achieved by Topactive Des. was 0.47 $\log_{10}$ CFU/well. Statistical analysis of means indicated that $\log_{10}$ reduction achieved by disinfectants treatment was not influenced by the cells morphotypes (RDAR, BDAR, PDAR, SAW), pathotypes or the amount of biofilm formation. The biofilm of cattle

and clinical EHEC strains showed no statistical differences in their tolerance to disinfectant intervention (Figure 3.7).

Table 3-4. Exposure of *E. coli* O26 biofilms to Topactive Des., Profoam and Kwiksan 22 disinfectants for 10 min

Pathotypes	Strains No.	10 min exposure to sanitisation treatment					
		PBS	Topactive Des.	PBS	Profoam	PBS	Kwiksan
EHEC	EC1113B	6.5 (±0.2)	6.5 (±0.5)	7.06 (±0.2)	6.27 (±0.5)	6.50 (±0.5)	6.50 (±0.5)
	EC1643B	6.34 (±0.4)	6.33 (±0.3)	6.56 (±0.4)	6.13 (±0.1)	6.68 (±0.2)	5.79 (±0.1)
	EC1857	6.3 (±0.5)	6.3 (±0.7)	6.77 (±0.4)	6.49 (±0.3)	6.90 (±0.2)	6.75 (±0.3)
	EC217	6.74 (±0.1)	6.3 (±0.5)	6.30 (±0.3)	6.30 (±0.3)	5.67 (±0.2)	5.20 (±0.4)
	EC3522	6.54 (±0.8)	6.5 (±0.5)	6.60 (±0.3)	6.43 (±0.3)	6.35 (±0.3)	4.93 (±1.4)
	EC3547A	6.93 (±0.3)	6.91 (±0.1)	6.25 (±0.4)	6.18 (±0.5)	6.21 (±0.6)	5.61 (±0.4)
	EC3652B	6.07 (±0.1)	5.87 (±0.4)	6.00 (±0.3)	6.00 (±0.1)	6.32 (±0.2)	6.17 (±0.8)
	EC3659B	6.63 (±0.3)	6.43 (±0.4)	6.37 (±0.4)	5.12 (±0.9)	6.23 (±0.2)	4.70 (±0.6)
	EC3671A	6.4 (±0.3)	6.4 (±0.4)	6.76 (±0.3)	5.77 (±0.6)	6.20 (±1.1)	6.20 (±1.0)
	EC3738B	6.76 (±0.4)	6.73 (±0.3)	6.27 (±0.3)	6.21 (±0.4)	6.23 (±0.7)	6.08 (±0.5)
	EC3743A	7.1 (±0.3)	7.1 (±0.2)	6.61 (±0.5)	6.50 (±0.3)	6.60 (±0.4)	5.72 (±0.9)
	EC478B	6.7 (±0.2)	6.7 (±0.2)	6.32 (±0.4)	5.92 (±0.5)	6.80 (±0.05)	6.26 (±0.5)
	EC674	6.82 (±0.2)	6.72 (±0.2)	6.74 (±0.5)	6.32 (±0.8)	6.65 (±0.5)	6.24 (±0.9)
	EC7B	7.0 (±0.3)	7.0 (±0.01)	6.75 (±0.1)	6.27 (±0.8)	6.40 (±0.4)	6.40 (±0.5)
	EC3213QH34	6.7 (±0.3)	6.7 (±0.3)	6.55 (±0.7)	6.14 (±0.8)	6.73 (±0.2)	6.69 (±0.03)
	EC4158QH1	7.0 (±0.3)	7.0 (±0.0)	6.84 (±0.2)	6.31 (±0.2)	6.88 (±0.2)	5.23 (±0.7)
	EC4159QH2	7.0 (±0.1)	7.0 (±0.05)	6.50 (±0.3)	6.43 (±0.5)	6.82 (±0.3)	5.56 (±0.4)
	EC4160QH3	6.0 (±0.4)	6.0 (±0.4)	6.46 (±0.3)	6.42(±0.3)	6.24 (±0.5)	5.76 (±0.02)
	EC4162QH5	7.0 (±0.2)	7.0 (±0.1)	6.93 (±0.2)	6.18 (±0.4)	6.52 (±0.3)	6.51 (±0.2)
	EC4163QH6	6.3 (±0.3)	6.3 (±0.3)	6.44 (±0.3)	6.22 (±0.5)	6.39 (±0.3)	6.10 (±0.5)
	EC4164QH7	4.93 (±0.2)	4.46 (±0.6)	4.33 (±0.4)	4.00 (±0.9)	5.11 (±0.4)	4.44 (±0.9)
	EC4165QH8	5.35 (±0.6)	5.27 (±0.5)	5.80 (±0.4)	5.50 (±0.6)	5.16 (±0.9)	4.20 (±1.0)
pEHEC	EC3983A	6 (±0.6)	6 (±0.3)	7.00 (±0.9)	6.50 (±0.2)	6.52 (±0.04)	6.11 (±0.8)
	EC3989A	7.29 (±0.1)	6.93 (±0.3)	7.20 (±0.1)	7.01 (±0.2)	7.12 (±0.01)	6.53 (±0.4)
	EC801	6 (±0.8)	6.0 (±0.3)	6.00 (±0.7)	6.00 (±0.7)	6.22 (±0.5)	5.69 (±0.6)
aEPEC	EC3457	6.07 (±0.3)	5.907 (±0.6)	6.35 (±0.5)	4.45 (±1.1)	6.70 (±0.3)	6.05 (±0.2)
	EC3727A	6.18 (±0.1)	6.068 (±0.01)	6.10 (±0.1)	6.10 (±0.2)	6.16 (±0.3)	5.26 (±0.4)
	EC3735A	6.5 (±0.3)	6.46 (±0.3)	6.41 (±0.3)	6.38 (±0.4)	6.00 (±0.)	6.00 (±0.3)
	EC3768A	6.021 (±0.27)	5.68 (±0.4)	6.00 (±0.3)	6.00 (±0.2)	6.11 (±0.5)	5.49 (±0.7)
	EC4013A	6 (±0.016)	6 (±0.00)	5.64 (±0.8)	5.50 (±0.5)	5.94 (±0.1)	4.49 (±0.1)
NTEC	EC3536B	6.58 (±0.8)	6.13 (±1.1)	6.19 (±0.3)	5.88 (±0.5)	6.46 (±1.1)	5.76 (±1.4)
	EC3946A	7.22 (±0.1)	7.07 (±0.1)	7.10 (±0.1)	7.10 (±0.03)	7.05 (±0.05)	7.00 (±0.0)

Biofilms were formed in polystyrene plates and data are shown as mean log CFU per well (±the standard errors of the means). The influence of disinfectants on biofilms was determined by enumerating viable cells remaining after treatment with 1% Topactive Des., 0.45% Kwiksan 22 (QAC) and 1% Profoam (QAC) and compared to that of PBS control. Results are averaged from at least 3 biological replicates.

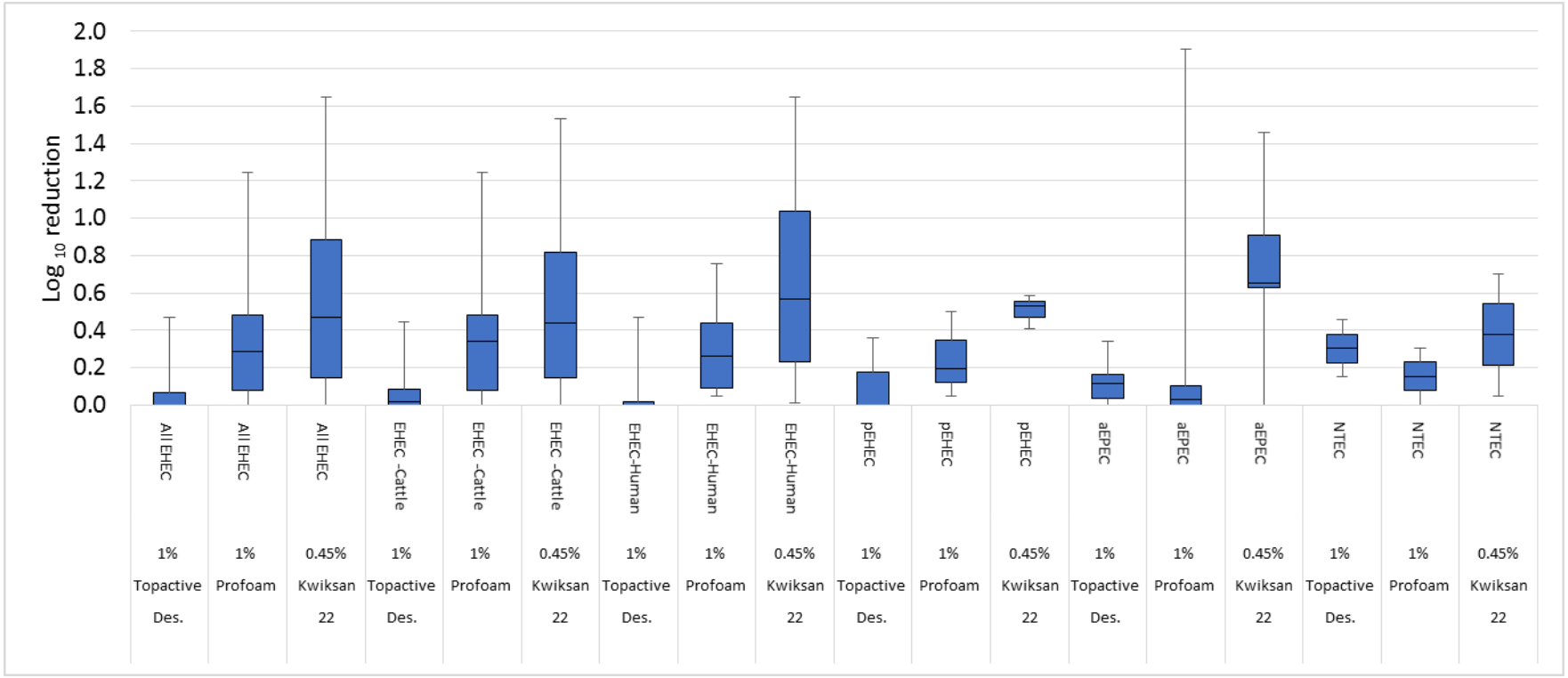


Figure 3-7. Box-plot of log_{10} reduction of *E. coli* O26 after 10 min exposure to 1% Topactive Des., 1% Profoam and 0.45 Kwiksan 22 based on strains source and pathotypes. For each box, the lower hinge, upper hinge and inside line represent the 25th (Q1) percentile, the 75th (Q3) percentile and the median, respectively. Lower and upper bars represent the lower and the upper whiskers respectively.

It’s noteworthy that exposure to disinfectants for 2 min led to higher mean log reduction and reduced viable cell counts of ≥ 1 $\log_{10}$ CFU/well than that of 10 min (Figure 3.8). For instance, after 2 min, 0.45% Kwiksan led to reduction of ≥ 1 $\log_{10}$ CFU/well viable cell counts of 12 EHEC strains and a single aEPEC strains whereas exposure to the same disinfectant but for 10 min resulted in reduction of ≥ 1 $\log_{10}$ CFU/well viable cell counts of four EHEC strains and a single aEPEC strain.

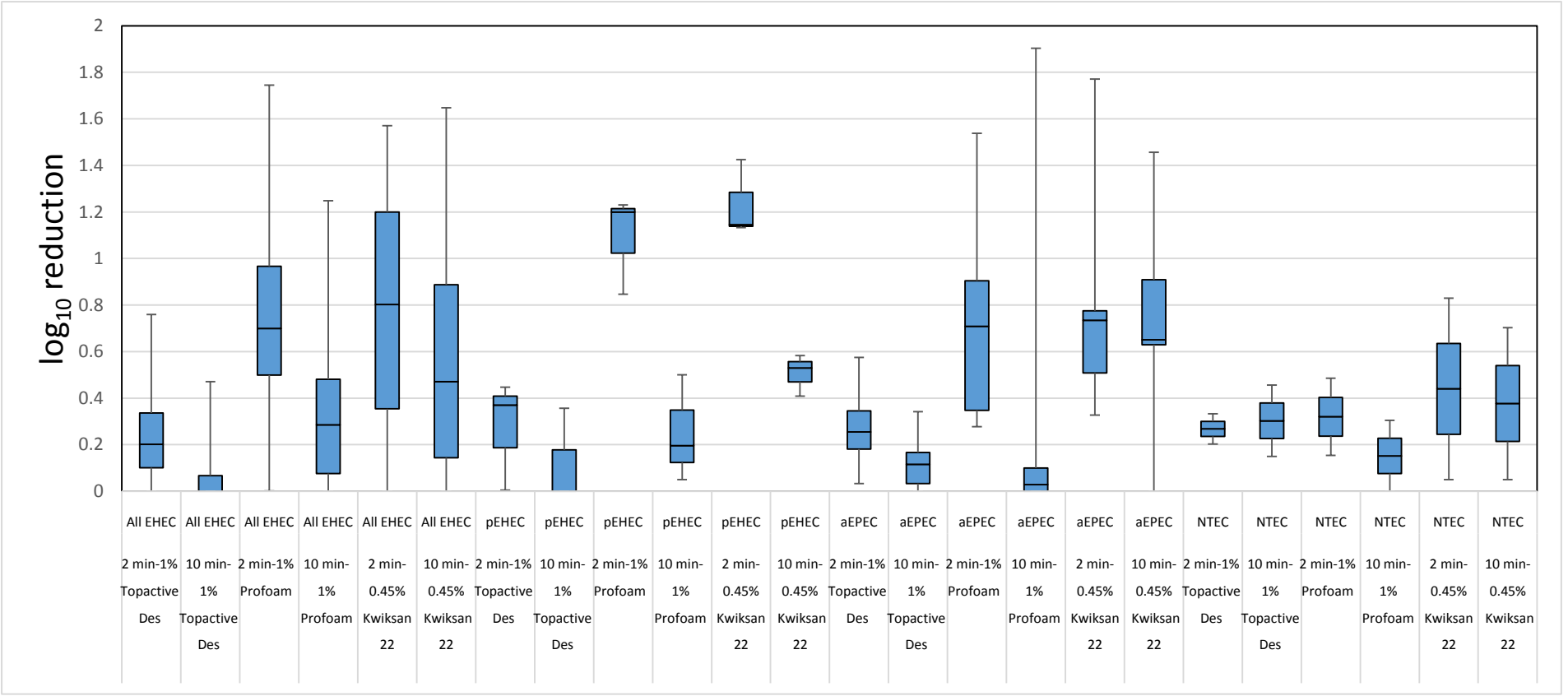


Figure 3-8. Box-plot of log_{10} reduction of *E. coli* O26 after 2 min and 10 min exposure to Topactive Des., Profoam and Kwiksan 22 based on pathotypes. For each box, the lower hinge, upper hinge and inside line represent the 25th (Q1) percentile, the 75th (Q3) percentile and the median, respectively. Lower and upper bars represent the lower and the upper whiskers respectively

3.10 **Discussion**

The role of autotransporter and fimbriae-encoding genes in biofilm formation and adhesion has been suggested previously (Low et al., 2006, Paton et al., 2001, Totsika et al., 2012, Vogeleer et al., 2015, Wells et al., 2008). Results presented in the current study did not identify any linkage between the presence of these genes and the ability to form biofilms. Vogeleer et al. (2015) demonstrated that autotransporter genes, *ehaB* and *espP*, and two fimbrial genes, *z1538* and *lpf2* (*loc13*), were potential genetic determinants for biofilm formation. However, in that study, strains were categorised as biofilm producing or non-biofilm producing at an OD level different from that used in this study. Overall, although it was not possible to identify an association between biofilm formation and prevalence of autotransporter genes and/or fimbrial genes, the distribution of these genes in EHEC O26 from the current study was consistent with that from the previous studies regardless of the capacity of biofilm formation (Vogeleer et al., 2015, Wells et al., 2008). As some of autotransporter genes and/or fimbrial genes were found in most of strains it is possible that they have a role in colonisation of the human gastrointestinal tract rather than colonising of abiotic surfaces. However, the role of these factors can only be elucidated using of knockout mutants.

The occurrence of RpoS mutation in *E. coli* O26 strains was investigated in this study. Previous reports suggested RpoS mutations may arise in response to environmental stresses such as nutrient depletion, acidic pH, oxidative stress, high temperature and osmotic stress (reviewed by Galhardo et al., (2007) and Lombardo et al., (2004)). The majority of EHEC strains (92.6%) in the current study had wild type RpoS and only two strains of EHEC pathotype (7.4%) had RpoS mutations. In contrast, previous studies identified that 26.3% and 72% of non-O157 strains and O157 respectively had RpoS

mutations (Chen et al., 2013, Uhlich et al., 2013). In this Chapter, although strains with RpoS mutations produced SAW morphotype and were limited in their capacity to form biofilm on glass slides and pellicle at the air-liquid interface, they formed moderate biofilm on polystyrene plate. Despite that the type of mutation was not investigated in this study, research suggest that these strains might carry RpoS with attenuated function (rather than inactivated RpoS) which would provide fitness advantage when stressful environmental conditions are encountered (Galhardo et al., 2007) or when occupying new niches.

QACs are used for medical disinfection and for sanitation in food-processing environments and cause cell death by inducing cytolytic injury leading to leakage of bacterial cellular components and adsorption of QACs compounds (Salton et al., 1951). Peracetic acid compounds oxidise the outer cell membranes of microorganisms leading to rapid deactivation of microorganisms (reviewed in Kitis (2004)). Application of QAC and peracetic acid based disinfectants did not completely remove or effectively reduce bacterial cells within a biofilm. This finding confirms that biofilm formation plays a role in providing protection against disinfectant intervention. However, unexpectedly, for some strains, mean $\log_{10}$ reductions caused by the tested sanitising agents after 2 min were greater than after 10 min of treatment regardless of disinfectant. This finding is unusual and cannot easily be explained. Further experiments using confocal laser scanning microscopy or fluorescent imaging methods to examine the interaction between disinfectants and biofilm matrix at these time points (2 min and 10 min) could elucidate the contradicted trend in the mean $\log_{10}$ reductions between the 2 min and 10 min exposure time (Bridier et al., 2011).

3.11 **Conclusion**

Results presented provided further insight into the characteristics of *E. coli* O26 biofilm forming strains. A comparison of autotransporter and/or fimbrial genes in biofilm and non-biofilm forming showed no association. RpoS mutation was not common among *E. coli* O26 strains as it was identified only in two clinical strains. Encasement of *E. coli* O26 cells within a biofilm provided a protective effect against all the disinfectant tested.

Chapter 4

Phenotypic and Genotypic Characterisation of Shiga Toxin 1 Bacteriophage of *E. coli* O26 from Clinical Cases and Cattle in Australia.

4.1 **Abstract**

Background: Enterohaemorrhagic *E. coli* (EHEC) O26 is a foodborne pathogen that can be found in cattle and can cause a range of human clinical diseases. The acquisition or loss of Shiga toxin (*stx*) which is carried on phage can alter the pathogenic potential of an *E. coli* strain. The aim of this study was to characterise Stx1-encoding phage of EHEC O26 and explore whether it's able to infect and persist in *stx* negative *E. coli* O26 strains.

Results: Stx1 bacteriophages insertion sites (SBIs) in 27 EHEC; and their status in *stx* negative (n=13) were investigated. In 12 *stx* negative strains, all loci (n=8) were vacant and in the remaining single isolate *yehV* was occupied. In EHEC, the following insertion sites were shown to be occupied by bacteriophage: *wrbA* in a single human isolate, both *yehV* and in vicinity of *torS* in seven isolates and in the vicinity of *torS* in 19 isolates. Inducing Stx1-encoding phage of EHEC O26 with mitomycin C (MMC) to lysogenise *stx* negative isolates and three K12 laboratory strains revealed that Stx1-encoding phage can lysogenise other bacterial strains transiently or permanently and integrate into a site within the vicinity of *torS*. Expression of *stx1* was quantified for a subset of spontaneously (NMMC) and MMC induced cultures for 3 and 6 hr using QuantStudio dPCR. Analysis revealed that *stx* expression levels were higher in MMC samples than NMMC either after 3 hr or 6 hr of induction. Evaluation of Stx1 production by ELISA for a subset of nine MMC induced cultures concluded that lysogens produced more Stx than parental strains. Induction levels vary between

isolates regardless of the strain origin and were not predictive for infectivity, toxin production or expression.

Conclusion: This study confirms that Stx1-encoding phage can transfer into other *E. coli* strains transiently or permanently and influences the levels of phage induction and Stx expression and production. The ability of Stx bacteriophage to infect and excise from *E. coli* O26 strains must be considered when attempting to detect causative agents of foodborne disease.

4.2 Introduction

E. coli can belong to a diverse group of pathotypes depending on the virulence markers they possess. Shiga toxin producing *E.coli* (STEC) is a pathotype associated with a variety of human clinical disease scenarios. A subset of STEC, known as enterohaemorrhagic *E. coli* (EHEC) has attracted significant attention because of its involvement in serious foodborne illnesses and association with severe bloody diarrhoea, haemolytic uremic syndrome (HUS) and haemorrhagic colitis (HC). EHEC can be transmitted to humans by contact with animals on farms, consumption of contaminated meat, beef sausage, milk and its derivatives, water, spinach, sliced watermelon, clover sprouts, blueberries and strawberries (Hiruta et al., 2001, Ethelberg et al., 2009, Hoshina et al., 2001, Lynch et al., 2012, CDC, 2012). Some EHEC serotypes such as O157 and non-O157 (O26, O45, O103, O111, O121, and O145) are classified as adulterants in some beef export markets. These serotypes have been linked with severe clinical illness in humans. Among those serotypes, *E. coli* O26:H11 is the most frequent non-O157 EHEC associated with serious foodborne outbreaks worldwide (CDC, 2015b, EFSA and ECDC, 2016a, Guth et al., 2002, Kanayama et al., 2015, Vally et al., 2012).

EHEC O26 strains harbour genes encoding Shiga toxins 1 and/or 2 (stx_1, stx_2) and the *E. coli* attaching and effacing gene *(eae*) (Allerberger et al., 2003, Guth et al., 2002). Both of these genes are usually found in clinical strains associated with human illness (Gerber et al., 2002). The gene, *stx*, is located in the genome of temperate bacteriophages and is recognised as the major virulence factor contributing to strains' pathogenicity and is used as a genetic marker for identification of EHEC from other pathotypes. Based on the antigenic and genetic variations, two major types of *stx* have

been identified: *stx1* and *stx2* (O'Brien et al., 1982, Strockbine et al., 1986) which can be expressed individually or together by EHEC strains. A number of variants exist within both *stx1* and *stx*2. *stx*1 consists of subtypes *stx1a*, *stx1c* and *stx1d* and *stx2* variants include *stx2c*, *stx2d*, *stx2e*, *stx2f*, *stx2g* (Scheutz et al., 2012) and *stx2dac*t (Bunger et al., 2013). Besides *stx*, strains of EHEC possess a pathogenicity island known as locus of enterocyte effacement (LEE) which encodes a type III secretion system (T3SS), regulators, chaperones, translocated and effector proteins (Jerse and Kaper, 1991). All of these proteins along with an adhesin called intimin which is encoded by *eae* participate in the formation of the attaching and effacing lesions (A/E lesions) (Hartland et al., 1999). The *eae* gene is used in combination with *stx* for detection of EHEC.

Similar to EHEC, atypical enteropathogenic *E. coli* (aEPEC) O26 strains which are a major cause of infant diarrhoea, harbour the LEE Pathogenicity Island and have the capacity to cause the A/E lesions but unlike EHEC, aEPEC strains do not produce Stx (Bielaszewska et al., 2007). The production and the release of Stx is a key factor in the pathogenicity of severe illness associated with EHEC such as HUS (Bielaszewska et al., 2006, Tyler et al., 2013). Isolation of EHEC O26 from patient stools early in illness and aEPEC O26 from stools of the same patients later in illness, and vice versa have been reported previously (Bielaszewska et al., 2007). A number of studies reported the conversion of *stx*-negative strains into *stx* positive strains and demonstrated acquisition of *stx in vivo* (Sekse et al., 2008) and *in vitro* (Iversen et al., 2015, James et al., 2001, Schmidt et al., 1999a) using labelled *Stx2*-encoding bacteriophage. Similarly, studies have demonstrated that Stx1 can be integrated into the genome of Stx-negative strains or K12 laboratory strains (Acheson et al., 1998, Bielaszewska et al., 2007, Bonanno et al., 2016). In the light of these studies, integration of *Stx*-encoding bacteriophage into

the bacterial host genome contributes to the evolution of a bacterial strain, giving rise to the emergence of a new pathogen as well as dissemination of Stx. The integration of phage into a bacterial host is mediated by a group of site-specific recombinase enzymes known as phage integrases which are classified as the tyrosine recombinases (λ integrase classified within this group) and the serine recombinases (Balding et al., 2005). A variety of Stx bacteriophage insertion sites (SBIs) have previously been identified for O157 and non-O157 strains. These include *wrbA, yehV, yecE, sbcB, Z2577, argW, prfC* and *torS-torT* (Steyert et al., 2012).

In Chapter 1, a number of *E. coli* O26 cattle strains were demonstrated to be highly related to EHEC, however they lack *stx* and as a result they were categorised as potential EHEC (pEHEC). Understanding the capacity for these strains to acquire *stx* and identifying where it may integrate is important as *stx* acquisition could enhance bacterial pathogenicity and impact pathogen evolution and thereby pathotype classification. In this study, EHEC O26 from human and cattle were assessed and compared for their SBI site status and their capacity to transduce K12 laboratory strains. In addition, the capacity of *stx*-negative O26 strains to acquire Stx and become EHEC was also assessed. Phage induction, infectivity level, host range, toxin production and expression were examined.

4.3 Materials and Methods

4.3.1 Bacterial strains

A total of 40 *E. coli* O26 strains characterised previously (Chapter 2 and Chapter 3; Table 3.1) in planktonic and biofilm state were utilised in this study. EHEC parent

strains were comprised of a total of 27 Australian *stx1 E. coli* O26 strains of which ten were isolated from clinical samples and 17 were isolated from cattle. Host or recipient strains consisted of control K12 laboratory strains (Q358, MG1655 and DHα5) and 13 *stx*-negative strains comprising: aEPEC O26 (n=8), pEHEC O26 (n=3) and non-toxigenic *E. coli* (NTEC O26; n=2).

4.3.2 Identification of SBIs

The chromosomal loci that serve as integration sites for Stx-encoding phages and the status of the insertion sites as intact or occupied for 40 *E. coli* O26 strains including 27 *stx1* positive and 13 *stx*-negative were interrogated using previously published primers for *wrbA, yehV, yecE, sbcB, Z2577, argW, prfC* and *torS-torT* [listed in (Bonanno et al., 2015)].

Lambda bacteriophage insertion sites were assessed bioinformatically using previously published primers (Balding et al., 2005). Genes adjacent to the integrase were identified using BLAST analyses (National Centre for Biotechnology Information, NCBI, Bethesda, USA). Sequence alignment was performed to identify integrases specific to EHEC. Additional primers were then designed (Table 4.1) using Primer 3 (Rozen and Skaletsky, 2000) to amplify the detected integrase and to determine whether the *torS* was located next to *torT* and was unoccupied or interrupted as well as to determine if the *torS* was adjacent to the detected integrase.

Table 4-1. Primers used for identification of additional SBIs for *E. coli* O26 strains

Purpose	Primers	Product size	Reference
Detect lambda integrase	F-GTTACMGGGCARMGAGTHG R- ATGCCCGAGAAGAYGTTGAGC	378 bp	Balding et al., 2005
Identify and detect integrase associated with	int-F-ACTGACATATCAACGAAAGAAG int-R: CGATTATAGTTTCACTGCTG	468bp	This study
Detect *torS*	torS-F: GAGTGCTGATTTCACTGTCCTG torS-R: AGCTCAATAATGCGGTGAAAAT	150 bp	This study
Detect *torT*	torT-F: CGTTGAACTATGGTATGCAGGA torT-R: TATCACCAAGCGCAA TATCAAC	438bp	This study
Determine if *torS-torT* intergenic region was unoccupied nor	torS-F: GAGTGCTGATTTCACTGTCCTG torT-R: TATCACCAAGCGCAA TATCAAC	1553	This study
Determine if *torS* is adjacent to the discovered	torS-F: GAGTGCTGATTTCACTGTCCTG int-F:ACTGACATATCAACGAAAGAAG	1672bp	This study

4.3.3 Stx-encoding phage induction and isolation

EHEC strains were grown in Luria Bertani (LB) broth at 37°C for 18 hr with rotary shaking at 250 rpm. Overnight cultures of EHEC were diluted in 50 ml tubes to 0.1 and were grown again at 37°C with rotary shaking at 250 rpm to OD_{600}, 0.3 to 0.5. When it reached the required OD, cultures were divided into two tubes. One was induced by adding Mitomycin C (MMC) (Sigma-Aldrich, Germany) (final concentration of 1µg/ml) and the other one was used as a control (NMMC) to evaluate the growth in the absence of MMC. After being incubated for 18 hr with rotary shaking, the rate of phage induction was evaluated spectrophotometrically by measuring the optical density (OD_{600}) of induced and non-induced cultures (Wagner et al., 2002, Muniesa et al., 2003). As MMC causes Stx- encoding bacteriophage induction which results in cell lysis, the decrease in the $OD._{600}$ of bacterial cultures is an indicative of phage induction, cell lysis and phage release. Strains were assigned into three categories based on their induction level: high: <0.4; Medium: >0.4 – 0.85; low: >0.85. Induced cultures were centrifuged at 4500 rpm for 10 mins at 4^{o}C, membrane filtered (Millipore; 0.45 µm pore

diameter) to isolate the phage lysate and the presence of *stx* in all EHEC parent cultures was confirmed by performing Paton Multiplex PCR (Paton and Paton, 1998) on the phage filtrate.

4.3.4 Spot agar test assay and susceptibility to infection

For this assay, cultures of *stx*-negative *E. coli* O26 strains of aEPEC, NTEC and pEHEC from cattle and K12 strains (MG1655, Q358 and DH5α) were diluted 1:100 and either grown in LB broth at 37°C with rotary shaking (250 rpm) to stationary phase (18 hr) or incubated at 37°C with rotary shaking (250 rpm) to reach to mid-exponential growth phase (OD_{600}, 0.3 to 0.5). Grown cells were then screened for susceptibility to lysis with Stx phage lysate by serially diluting MMC induced phages (10^{-1} to 10^{-5}) followed by spotting 20 µl of each dilution onto LB agar overlaid with 4 ml LB soft agar (0.35%) containing 100 µl of recipient cells (exponential or stationary phase *stx*-negative *E. coli* O26 strains or *E. coli* K12 strains) with sterile 10 mM $CaCl_2$ (final concentration). Plates were examined for their susceptibility to Stx phage infection after overnight incubation at 37^{o}C or incubating at room temperature (18-22^{o}C) for 72 hr. This was indicated by the occurrence of either clear lysis on *stx*-negative *E. coli* O26 strains and K12 strains used as a lawn, or turbid plaques of incomplete lysis or colonies within the zone of Stx phage lysis. Colonies that grew in the lysis area were screened by Paton PCR (Paton and Paton, 1998) for the presence of *stx*.

4.3.5 Construction of lysogens

Construction of lysogens was performed as described by Sim et al. (2015) with modifications. Briefly, a 3mL aliquot of phage lysate was added to 10 ml cultures of

stationary or mid exponential phase recipient strains that had been grown in the presence of 10 mM $CaCl_2$. Cultures were further incubated without agitation for 72 hr at 37^{o}C. At the end of the incubation, the host-phage mixture was centrifuged at 4500 rpm at 4^{o}C for 10 mins, supernatant was discarded and the pellet was used to detect Stx acquisition. Pellets that tested positive for *stx* were considered positive for lysogen formation and recovery of the lysogens was attempted.

4.3.6 Detection of *stx* acquisition from the lysogenic infection

Two methods were used to detect Stx phage acquisition. In the first method, each pellet was suspended in 1 ml LB broth, serially diluted (1:10) and a spread plate was performed (Sim et al., 2015). After overnight incubation, 20 to 60 colonies from plates with well-separated colonies were screened by PCR for *stx.* This method was used only for detection of lysogen generated from infecting stationary phase recipients.

The second method used colony hybridisation and hydrophobic grid membrane filters (HGMF) to identify colonies of interest as outlined by Barlow et al., (2004). Lysogen pellets were resuspended in 1 ml of PT diluent (1 mL of 0.1% peptone supplemented with 1% Tween 80) and serially diluted to $0.5x10^{-5}$ in PT diluent. The prepared dilutions were filtered through HGMFs on a spread filter (Filtaflex, Canada) using a vacuum manifold. HGMFs were then transferred onto LB agar and incubated for 18 ± 2 hr at 37°C. At the end of the incubation colonies were replicated onto a fresh HGMF using the HGMF replicator inoculator system (Filtaflex, Canada) and incubated as mentioned above. The membranes were soaked individually in pre-treatment solution (Yan et al., 1996) and were then subjected to colony lysis (Nizetic et al., 1991). Lysis was followed by pre-hybridisation at 68°C for 1 hr in hybridisation solution before

incubating the membranes overnight with Karch and Meyer (1989) DIG-labelled PCR probe (Roche Diagnostics, Germany). Finally, detection of the labelled probe was performed at room temperature with gentle shaking using Anti- Digoxigenin AP Fab fragments and the detection reagent NBT/BCIP solution following the manufacturer's protocol (Roche Diagnostics, Switzerland).

Observation of a purple dot at the site of the hybridisation indicates the presence of presumptive *stx* positive colonies which were subsequently picked and streaked onto LB agar. The acquisition of Stx phage was confirmed by performing Paton multiplex PCR (Paton and Paton, 1998) on the presumptive *stx* positive colonies. The stability of the phage containing lysogens from both methods 1 and 2 was evaluated by subculturing lysogens on LB agar for three passages and likewise, after each passage, lysogens were tested by PCR for the presence of *stx*.

4.3.6.1 Identification of SBIs for lysogens

The SBI status of lysogens was determined using the methods outlined in section 4.2.2 (Materials and Method). K12 laboratory strain was used as a control when appropriate.

4.3.7 *Stx* expression by QuantStudio digital PCR (dPCR)

EHEC parent strains that produced a stable lysogen were grown overnight at 37°C in sterile LB broth with shaking. Grown cultures were diluted to 0.1 at OD_{600} and then further incubated with rotary shaking (250 rpm) at 37°C to an OD_{600} of 0.3-0.5 followed by induction with a final concentration of 0.5 µg/mL MMC for 3 hr for all parent strains and 6 hr for a subset of strains based on source, PFGE cluster (Chapter 1) and Stx induction levels (This chapter). Following each MMC induction time (3 or 6 hr),

cultures were stabilised by adding 2 volumes of RNAprotect Bacteria Reagent (QIAGEN, Germany) to one volume bacterial culture, mixed immediately and then incubated for 5 min prior to centrifugation for 10 min at 5000 x g. The obtained pellet was then used for RNA extraction using a Promega SV total RNA purification kit (Promega; Alexandria, Australia) according to the manufacturer's instructions. RNA was finally eluted in 100 µL of nuclease free water and its integrity and quality were checked using the TapeStation system (Agilent, USA) using the RNA ScreenTape assay. The RNA Integrity Number (RIN^e) is calculated automatically on a scale from 1 (low) to 10 (high) with a low RIN^e indicating a strongly degraded sample. All assays were performed as three independent biological replicates.

To purify the RNA and remove the residual DNA, samples were subsequently treated with RNase-free DNase I (Turbo DNA-free; Ambion, TX). RNA concentration was confirmed with the Qubit 2.0 fluorometer (Invetrogen, Life Technologies, Singapore). RNA template was normalised in order to have 250 ng of RNA in each cDNA sample. Four samples were produced for each strain. These included: MMC induced with reverse transcriptase (RT), and spontaneously induced (NMMC) with RT. Additionally, each MMC and NMMC cDNA was prepared without reverse transcriptase (NRT). The comparison between MMC and NMMC induced in the presence of RT enabled discrimination between basal and induced levels of *stx*. Further, the reactions without RT (NRT) are included to confirm the absence of genomic DNA contamination. cDNA Synthesis was performed with iScript (Bio-Rad, Australia) as three independent biological replicates in 20 µl reactions containing 250ng cDNA template and 1 X iScript master mix with RT and without RT. (NRT). An aliquot of 2 µl of resulting cDNA from each sample (RT MMC, RT NMMC, NRT MMC, and NRT NMMC) or post synthesised cDNA samples diluted 1:10 and 1:100 (RT MMC) were added to 12.5 µl

master mix and loaded onto dPCR chips. The dPCR chips were loaded into the Proflex PCR system (Applied Biosystems, life technology, USA) and PCR conducted according to the manufacturer's instruction as follows: stage 1; 96^{o}C for 10 min, stage 2; 39 cycles of 60^{o}C for 2 min and 98^{o}C for 30 sec, stage 3; 60^{o}C for 2 min and hold at 10^{o}C. To calculate the absolute *stx* copy number, subsequent analysis of the chips were performed online using the QuantStudio™ 3D analysis software (https://apps.thermofisher.com/quantstudio3d/projectHome .html). The Poisson equation is used by QuantStudio software to calculate target quantity from dPCR data.

4.3.8 Total Stx toxin production using ELISA assay

Comparative assessment of total Shiga toxin production was performed on the subset of strains that were able to make a lysogen and retain *stx* genes after three subsequent passages on LB agar. Shiga toxin production levels of MMC induced cultures was assessed using Premier EHEC enzyme-linked immunosorbent assay (ELISA) (Meridian Bioscience Inc., USA) with cultures prepared and diluted according to the method described by Mellor et al. (2015). The ELISA was used to determine the total Shiga toxin production level for each strain in duplicate following the manufacturer's instructions. Absorbance values were obtained at dual wavelengths (450 nm/630 nm) using a Victor X microtiter plate reader (PerkinElmer, Australia). Strains were assigned into three groups and scored based on the mean Stx production: low: <1; Medium: >1 – 2; high: >2 – 3 with three strains were used as a control: MG1655 is the negative control, EC3727 and EC1596-EDL933 are positive controls which have previously shown to produce medium level and high level of Stx respectively (Mellor et al., 2015).

4.4 Results

4.4.1 Identification of SBIs for *E. coli* O26 strains

A total of 27 EHEC and 13 *stx*-negative strains were screened by PCR to identify SBI status (Table 4.2). *yehV* was identified as a prophage integration site in seven EHEC (EC1, EC4, EC3738B, EC4158QH1, EC4159QH2, EC4161QH4 and EC4165QH8). In one additional EHEC strain, namely EC4166QH9 Stx prophage was integrated into *wrbA.* None of the following tested loci *yehV, wrbA, sbcB, argW, yecE, prfC* and *torS*-torT, appeared to be occupied with Stx1 prophage in the remaining EHEC strains. In *stx*- negative strains all the tested loci were shown to be intact except in a single aEPEC (EC4039A) which displayed a prophage insertion at *yehV*.

Utilising degenerate primers (Balding et al., 2005), originally developed to identify lambda integrases associated with Enterobacteriaceae in environmental samples, an integrase associated with all EHEC except EC4166QH9 was identified bioinformatically [using whole genome sequence data for parent strains (Chapter 4). Genes adjacent to the integrase were identified as *torS* bioinformatically. Since the *torS* integrase was only observed in EHEC strains it was designated as the phage insertion site for *stx1* in *E. coli* O26.

In light of the previous results, additional PCR was performed to amplify the *torS-torT* intergenic region using torS-F/torT-R. However, EHEC strains generated a 1553 bp amplicon which was similar to the product size generated by *stx*-negative strains and the control strain *E. coli* O26:H11 str. 11368 indicating that the intergenic region is not interrupted. To test whether the integrase was located adjacent to *torS*, primers (torS-

F/int-F) were successfully used to amplify a 1672 bp amplicon that demonstrated that the integrase site is in the vicinity of *torS*. No segregation based on SBI data was observed between clinical and cattle strains.

4.4.2 Phage induction of clinical and cattle *E. coli* O26 strains

Stx phage induction levels were measured spectrophotometrically at 600 nm (Table 4.2) in the presence and absence of MMC. Results demonstrated that strains were diverse in their susceptibility to MMC with nine strains poorly inducible (OD $\geq$ 0.85), 12 strains were moderately inducible (OD < 0.85) and six strains were highly inducible. When induced phage were filtered to isolate Stx phage particles and PCR screened for the presence of *stx*, all the induced cultures released *stx*-encoding phage which was detectable by conventional PCR indicating successful Stx phage induction.

4.4.3 Spot agar test assay and susceptibility to infection

In comparison to the stationary phase grown recipients which exhibited resistance to infection by the phage particles, recipient cultures in the exponential growth phase were more susceptible to Stx phage particles with no difference in the susceptibility to human and cattle originated Stx phage observed. However, regardless of susceptibility, screening colonies by PCR for *stx* confirmed the absence of *stx* from all strains except ΦEC3659 which generated an unstable lysogen in Q358. No differences in the plaque sizes or morphologies were observed between plates incubated at 25 or 37^{o}C.

Table 4-2. Level of Stx phage induction measured spectrophotometrically at OD600, lysogen generated and occupied loci

Pathotype	strains	Source	Occupied loci	OD_{600}[1]		Host	Lysogen stability	SBI of lysogens
				NMMC	MMC			
EHEC	EC1A	Cattle	*torS*, *yehV*	1.41±0.001	0.86±0.03	No		
	EC1113B	Cattle	*torS*	1.40±0.001	0.50±0.05	No		
	EC1643B	Cattle	*torS*	1.38±0.07	1.021±0.00	No		
	EC1857	Cattle	*torS*	1.40±0.003	0.99±0.009	No		
	EC217	Cattle	*torS*	1.41±0.003	0.41±0.004	No		
	EC3455	Cattle	*torS*	1.42±0.002	1.13±0.02	Q358	Unstable	*torS*
	EC3522	Cattle	*torS*	1.48±0.006	0.45±0.02	No		
	EC3547A	Cattle	*torS*	1.40±0.004	1.01±0.07	No		
	EC3652B	Cattle	*torS*	1.43±0.009	0.34±.0.05	Q358, DH5α	Stable, Unstable	*torS*
	EC3659B	Cattle	*torS*	1.45±0.004	0.46±0.05	Q358	Stable, Unstable[3]	*torS*
	EC3671A	Cattle	*torS*	1.41±0.002	0.58±0.02	No		
	EC3738B	Cattle	*torS*, *yehV*	1.05±0.008	0.50±0.006	No		
	EC3743A	Cattle	*torS*	1.41±0.01	0.25±0.05	Q358	Stable[4]	*torS*
	EC4	Cattle	*tors*, *yehV*	1.45±0.004	0.31±0.07	No		
	EC478B	Cattle	*torS*	1.41±0.01	0.39±0.05	Q358	Stable	*torS*
	EC674	Cattle	*torS*	1.41±0.003	0.55±0.2	No		
	EC7B	Cattle	*torS*	1.38±0.02	1.14±0.02	Q358	Unstable[4]	*torS*
	EC4158QH1	Clinical	*torS*, *yehV*	1.38±0.008	0.60±0.01	No		
	EC4159QH2	Clinical	*torS*, *yehV*	1.46±0.01	0.42±0.1	MG1655, Q358	Stable, Unstable	*torS*
	EC4160QH3	Clinical	*torS*	1.43±0.009	0.53±0.04	DH5α	Unstable	*torS*
	EC3213QH34	Clinical	*torS*	1.37±0.01	1.11±0.008	No		
	EC4161QH4	Clinical	*torS*, *yehV*	1.43±0.008	0.77±0.008	Q358	Stable	*torS*
	EC4162QH5	Clinical	*torS*	1.39±0.01	0.32±0.04	Q358, EC3610[5]	Stable, Unstable	*torS*
	EC4163QH6	Clinical	*torS*	1.44±0.006	1.12±0.01	Q358	Stable	*torS*
	EC4164QH7	Clinical	*torS*	1.41±0.005	1.41±0.01	No		
	EC4165QH8	Clinical	*torS*, *yehV*	1.41±0.01	0.7±0.04	Q358, MG1655	Stable, Stable	*torS*
	EC4166QH9	Clinical	*wrbA*	1.43±0.01	0.38±0.008	No		
	STX1 cocktail	Cattle	*torS*	ND[6]	ND	Q358	Stable	*torS*
	STX1 cocktail	Clinical	*torS*	ND	ND	Q358	Stable	*torS*
aEPEC	EC4039A	cattle	*yehV*					

[1]High phage induction level results in cell lysis and decrease in the cultures' optical density whereas an increase in the cultures optical density indicates bacterial growth due to low cell lysis and low prophage induction.[2] Data are shown as mean of induction (±the standard errors of the means). [3]EC3659 generated two unstable lysogens one was detected by PCR from screening colonies grown agar spot method and the other one was detected by mixing phage lysate with exponential grown host and detected by HGMF. [4]Lysogen was detected from infecting stationary phase grown recipients by picking 20 to 60 colonies from plates with well-separated colonies. [5]EC3610 is aEPEC strain. [6]ND: Not determined. Other lysogens listed in the table were detected by HGMF methods. aEPEC, pEHEC and NTEC strains that were negative for all tested loci (loci *yehV*, *wrbA*, *sbcB*, *argW*, *yecE*, *prfC* and *torS*-torT) are not shown in the table.

4.4.4 Lysogenic infection

4.4.4.1 Lysogenic infection in K12 strains.

Induced phage filtrates were used to lysogenise K12 strains (Q358, MG1655 and DH5α). All bacterial strains used to generate lysogens and list of bacterial hosts used in this assay and generated lysogens are listed in Table 4.2. Overall 12 stable lysogens were formed in K12 strains (Q358 and MG1655). Ten were generated using single phage lysate, with clinical strains more likely to form lysogens (six lysogens) than cattle strains (four lysogens) (Table 4.2).

4.4.4.2 Lysogenic infection in O26 *stx*-negative strains

Using induced phage lysate, no stable lysogens were generated in O26 *stx* negative strains (pEHEC, NTEC and aEPEC) and only one unstable lysogen was generated in a single aEPEC (Table 4.2). Despite repeated attempts, the transfer of *stx1* from any of the EHEC O26 strains to *stx*-negative O26 strains was unsuccessful. In addition, transduction of *stx*-negative O26 with *stx2* from *E. coli* O26 and a number of Argentinean *E. coli* O157 strains (Mellor et al., 2012) was performed (data not shown) but again it was not successful.

4.4.5 Identification of SBI for lysogens

Attempts were made to identify SBI for lysogens using previously targeted sites including the *torS-torT* intergenic region and the newly discovered loci (next to *torS*) (Table 4.2). In all lysogens, Stx bacteriophage was shown to be inserted next to *torS* similar to the SBIs of

parent strains. Since integrase adjacent to *torS* was identified only in EHEC and in lysogens it was designed as SBIs of EHEC however the *stx1* prophage boundaries remain undetermined.

4.4.6 *Stx* expression by QuantStudio dPCR

To test the level of *stx* expression by QuantStudio dPCR, Stx- phage induction at OD_{600} was measured spectrophotometrically after 3 and 6 hr incubation with and without MMC (Figure 4.1). Low induction levels as indicated by an increase in the cultures optical density due to low cell lysis were observed in NMMC tubes in comparison to MMC treated cultures with the majority of the strains showing greater induction (indicated by a decrease in the cultures optical density due to high cell lysis caused by phage induction) after 6 hr than 3 hr.

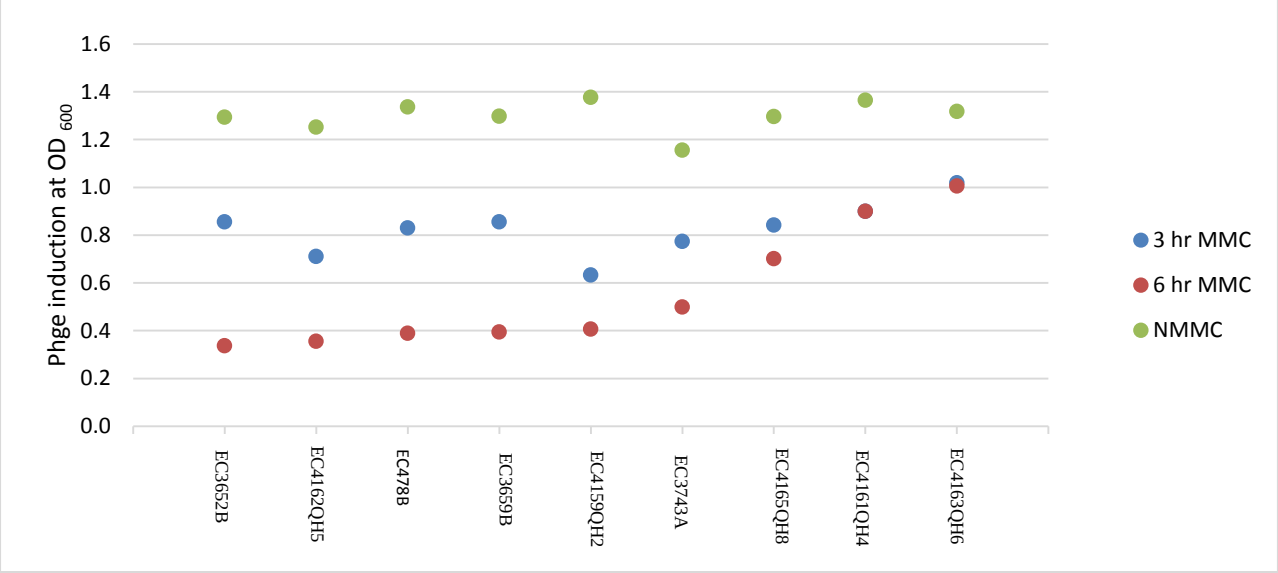


Figure 4-1. Levels of Stx phage induction were measured at OD_{600} in the absence of mitomycin C (NMMC) as well as 3 and 6 hr after adding mitomycin C (MMC). A decrease in the cultures optical density (OD. 600) was observed due to cell lysis caused by high phage induction level.

dPCR was used to measure the s*tx* copy number μl^{-1} after 3 hours of incubation in the presence and absence of MMC. Nine parent strains that had previously generated lysogens were assessed by this method (Figure 4.2). As expected, *stx* expression levels were higher in MMC samples with expression levels ranging from $\log_{10}$ 3.32 to 4.01 gene copies μl^{-1} compared to $\log_{10}$ 2.23 to 3.02 gene copies μl^{-1} for NMMC samples. The clinical strain EC4162QH5 produced ≈1.6 gene copies μl^{-1} fold more *stx* than all other strains, however no significant differences in average *stx* expression was observed between the remaining clinical ($\log_{10}$ 3.76 gene copies μl^{-1}) and cattle ($\log_{10}$ 3.83 gene copies μl^{-1}) strains.

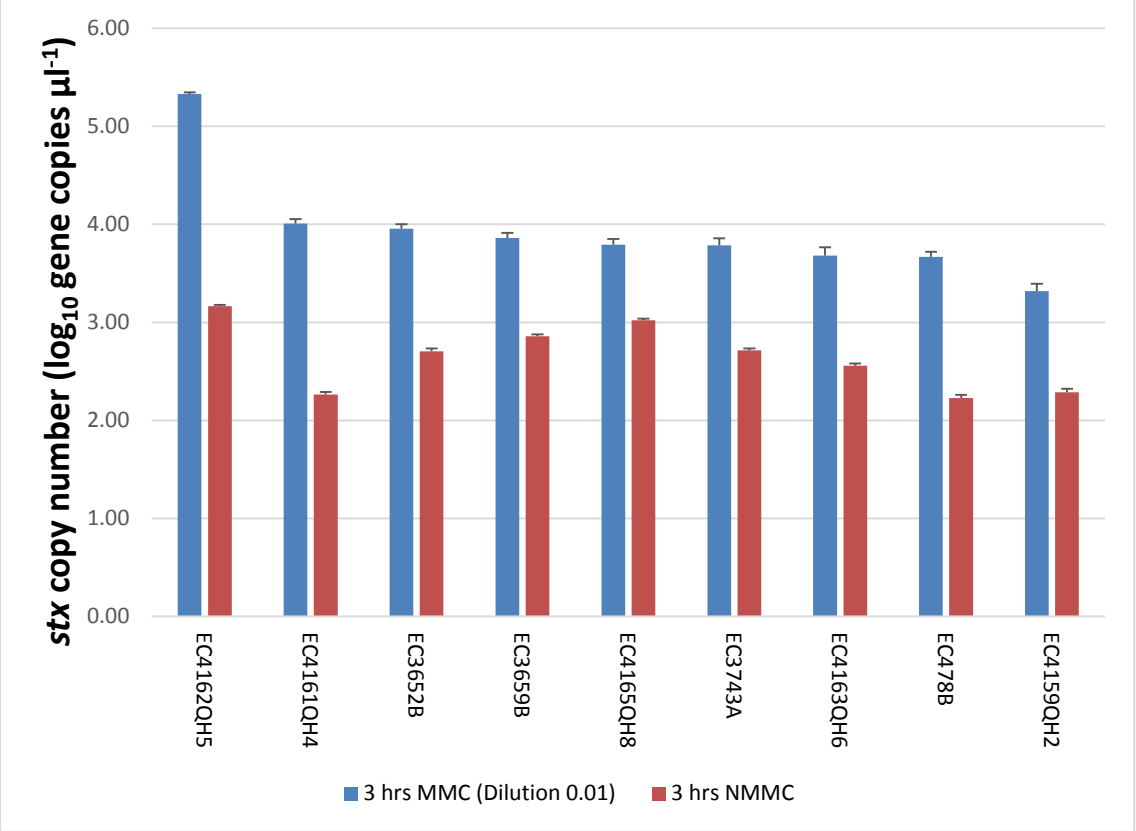


Figure 4-2. The average of *stx* expression ($\log_{10}$ gene copies μl^{-1}) by EHEC parent strains using dPCR in the absence of mitomycin C (NMMC) as well as after 3 hr of incubating exponential grown cultures with mitomycin C (MMC). The error bars represent the maximum and minimum Poisson distribution for the 95% confidence interval generated by the QuantStudio ™ software.

A subset of strains (EC4161QH4, EC478B and EC4159QH2) was assessed for *stx* expression level following 6 hr of NMMC and MMC induction (Figure 4.3). Results demonstrated that

stx expression levels in the presence of MMC after 6 hr were higher than that of NMMC; ranging from $\log_{10}$ 4.8-5.7 gene copies μl^{-1} compared to $\log_{10}$ 1.9-3.2 gene copies μl^{-1} of NMMC.

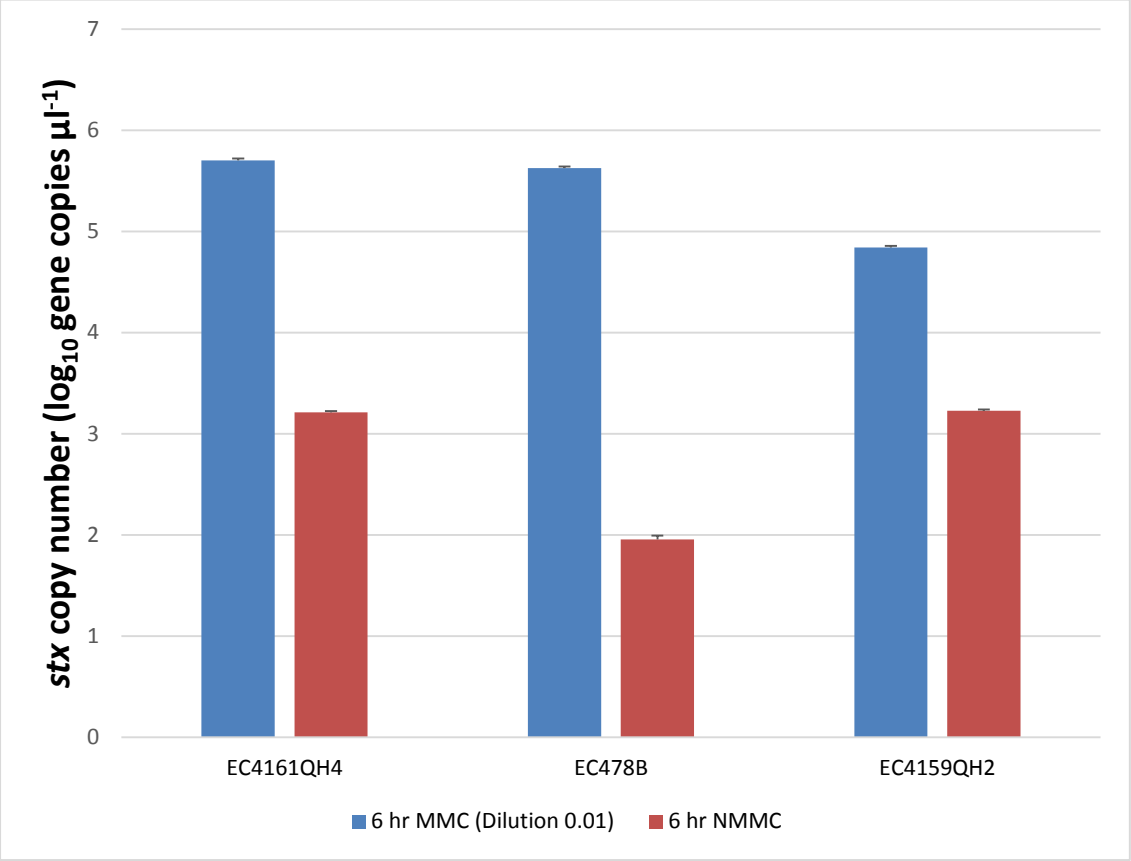


Figure 4-3. The average of *stx* expression ($\log_{10}$ gene copies μl^{-1}) by EHEC parent strains using dPCR in the absence of mitomycin C (NMMC) as well as after 6 hr of incubating exponential grown cultures with mitomycin C (MMC). The error bars represent the maximum and minimum Poisson distribution for the 95% confidence interval generated by the QuantStudio ™ software.

Comparing *stx* expression ($\log_{10}$ gene copies μl^{-1}) levels using dPCR in the absence of MMC and following exposure to MMC for 3 and 6 hr for a subset of strains namely (EC 4161QH4, EC478B and EC4159QH2) as shown in Figure 4.4. After 6 hr of MMC induction, strains showed a maximum of 1.4 fold increase in *stx* concentration in comparison to 3 hr. Similarly, NMMC induced samples for 6 hr exhibited higher *stx* level than 3 hr NMMC.

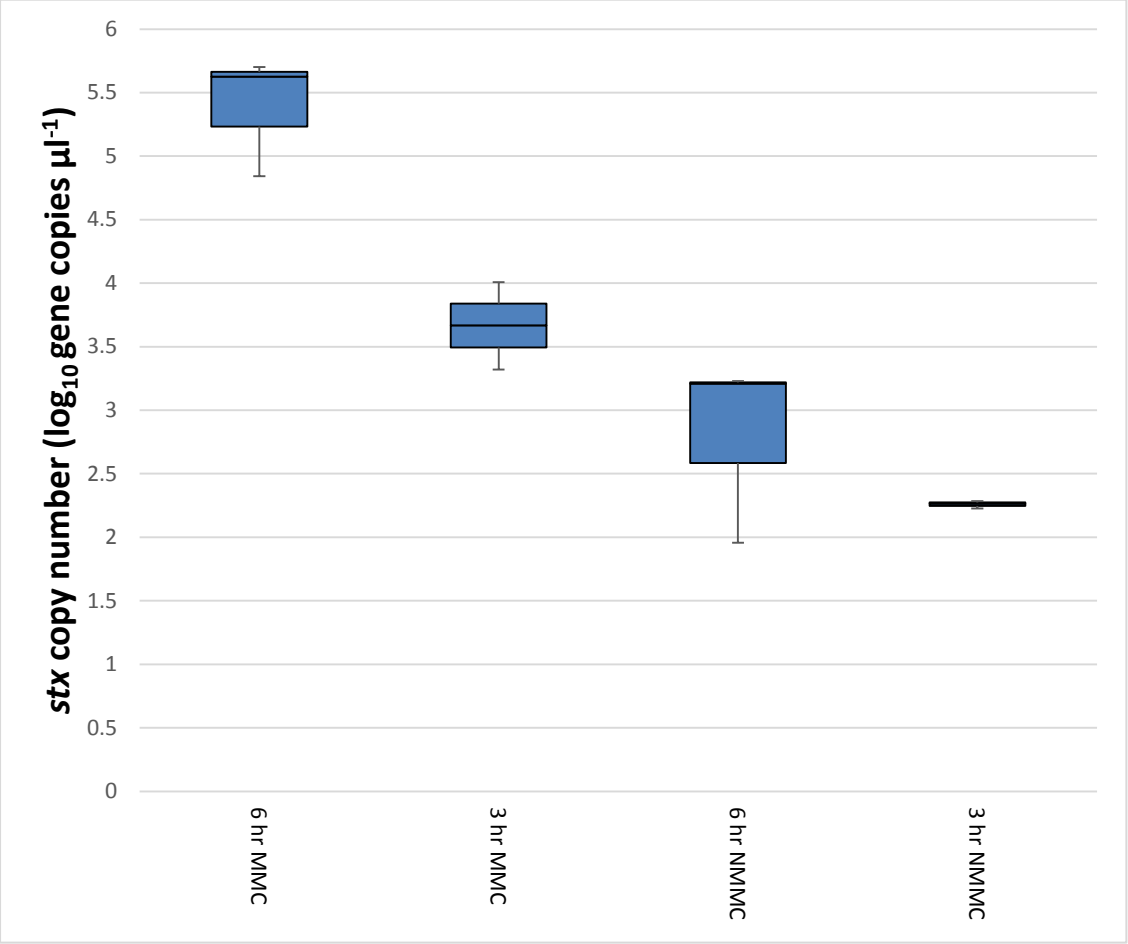


Figure 4-4. Box-plot of *stx* expression (log_{10} gene copies μl^{-1}) as measured by dPCR for three EHEC parent strains in the absence of mitomycin C (NMMC) as well as after 6 and 3 hr of incubating exponential grown cultures with mitomycin C (MMC). For each box, the lower hinge, upper hinge and inside line represent. For each box, the lower hinge, upper hinge and inside line represent the 25th (Q1) percentile, the 75th (Q3) percentile and the median, respectively. Lower and upper bars represent the lower and the upper whiskers respectively. 6 hr MMC and 3 hr MMC represent samples diluted 1:100.

4.4.7 Stx toxin production using ELISA assay

Stx production levels of nine *stx* parent strains and nine lysogens that retained *stx* after three subsequent passages on LB agar were estimated using the Premier ELISA assay. The subset of 18 strains tested showed differences in the level of *stx* production between *stx* parent strains and lysogens (Figure 4.5).

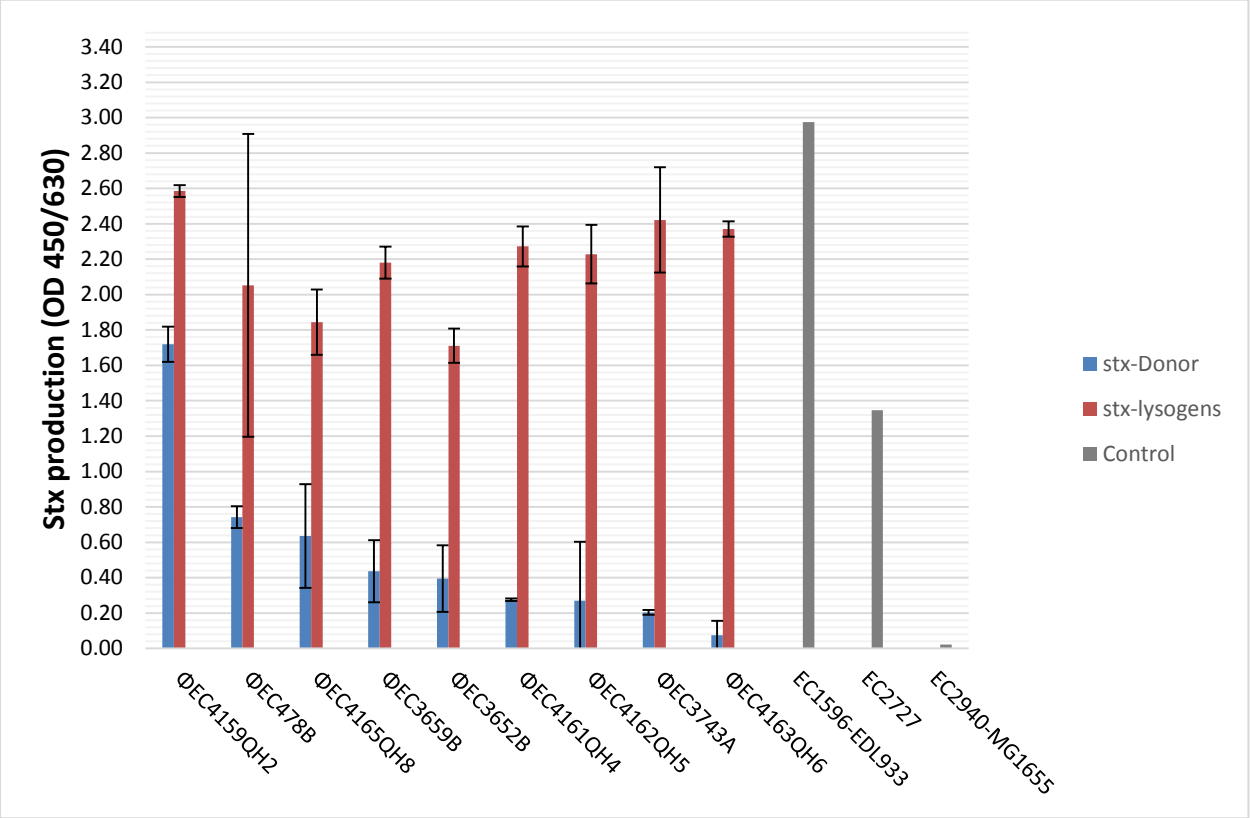


Figure 4-5. Stx production by Stx parent strains and lysogens using the Premier EHEC ELISA. Stx production measurements by ELISA were done in the presence of MMC. Values are the mean of independent experiments done in duplicate. Lysogens were all generated in Q358 except EC4159QH2 which generated a lysogen in MG1655. QH strains are clinical strains. Bars represent standard error (SE).

The production of Stx toxin by parent cells ranged from 0.07 to 1.72. In contrast, lysogens produced greater quantities of Stx with mean absorbance values ranging from 1.71 to 2.59 (Figure 4.6). No linear relationship (r=0.256) was found between the Stx production levels in the EHEC parent and lysogens strains. Differences between Stx production of clinical and cattle strains were not observed for either the parent strains (0.59 and 0.44 respectively) or lysogens (2.26 and 2.09 respectively).

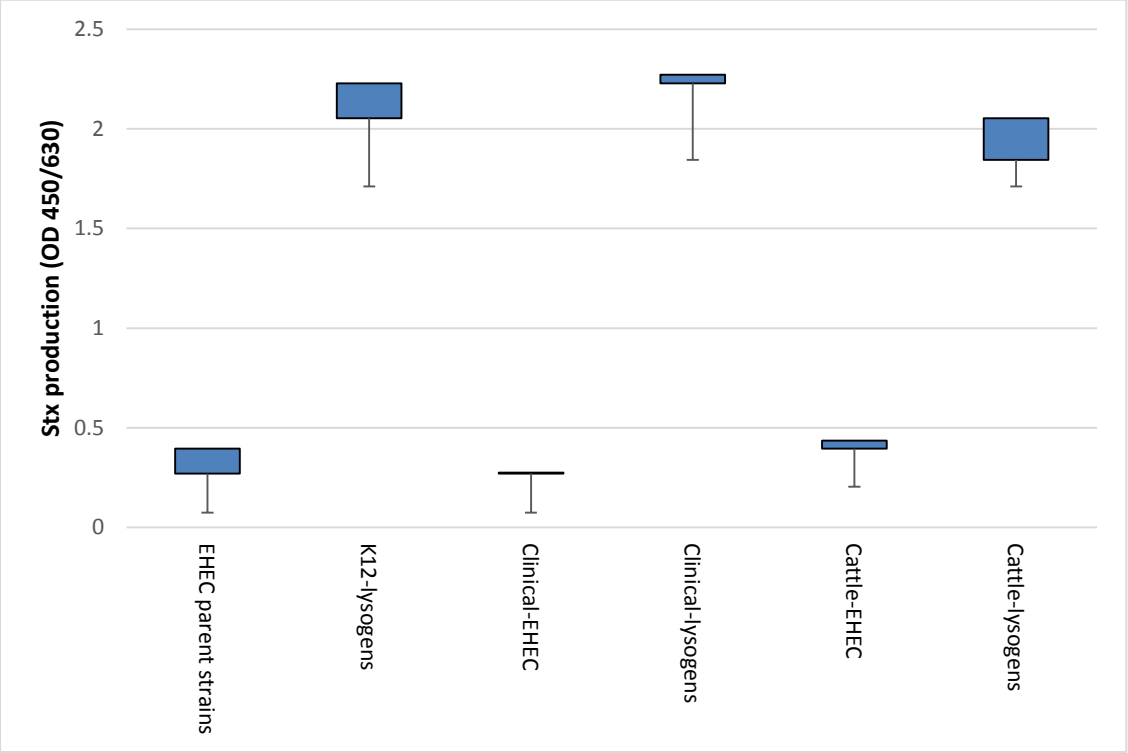


Figure 4-6. Box-plot of Stx production of *E. coli* O26 strains grouped by parent and lysogens and source. For each box, the lower hinge, upper hinge and inside line represent. For each box, the lower hinge, upper hinge and inside line represent the 25th (Q1) percentile, the 75th (Q3) percentile and the median, respectively. Lower and upper bars represent the lower and the upper whiskers respectively.

4.5 **Discussion**

E. coli O26 can belong to a range of pathotypes depending on the type of virulence genes they carry. *stx* is an important virulence factor and along with *eae* is regularly found in EHEC O26 strains recovered from foodborne disease cases (Ethelberg et al., 2009, EFSA and ECDC, 2016b). *stx* is carried on a phage whose acquisition or loss can alter the pathogenic potential of an *E. coli* strain. This study was conducted to initially determine the sites at which Stx phage integrate into the *E. coli* chromosome and to subsequently determine if Stx1-encoding phage can infect other *stx*-negative *E. coli* strains. Finally, the levels of Stx production in human and cattle strains and their lysogens were assessed.

In this study, the Shiga toxin bacteriophage of both human and cattle EHEC strains and corresponding lysogens were predominantly found to be inserted in a novel loci adjacent to *torS* but not in the previously mentioned *torS-torT* intergenic region of *E. coli* O103 (Ogura et al., 2007). This finding is novel for O26 and suggests that strains in Australia possess a SBI genotype different from human and cattle strains in other countries; however, the *stx1* prophage boundaries remain undetermined. Current approach based on PCR identification of genes in the vicinity of integrase has technical limitations and associated with difficulty in SBI characterization as observed in this study and other studies (Serra-Moreno et al., 2007, Bielaszewska et al., 2007, Mellor et al., 2012). The advances in next-generation sequencing are promising in elucidating the Shiga toxin insertion sites and complementing gaps in the current knowledge. Shiga toxin bacteriophage was also found to integrate into both *yehV* and next to *torS* in seven EHEC strains. Integration of Stx phage into *yehV* has been reported for *E. coli* O26 (Bonanno et al., 2016). However, regardless of where the phage was inserted in the parent strain and all lysogens had a phage inserted near *torS* but not *yehV*. This suggests that either *yehV* loci was occupied by non Stx prophage or that the EHEC strains carried more than one Stx prophage (Serra-Moreno et al., 2007). Finally, the Stx1 phage of a clinical strains was found to be inserted in the *wrbA* locus; an insertion site reported for *stx1* (Ogura et al., 2007) and *stx2* clinical strains (Bielaszewska et al., 2007). Further investigations to verify this finding by Stx transduction was not possible as this strain did not form a lysogen in either K12 strains or *stx*-negative strains.

Stx is the main virulence factor thought to be responsible for the severe outcome reported due to EHEC associated with human infection (Bielaszewska et al., 2006, Tyler et al., 2013). In this study, induction of Stx-encoding phage with MMC, an inducing agent that activates the SOS response, was measured spectrophotometrically and was shown to be variable (low,

medium, high) between strains. Nevertheless release of Stx-bacteriophage was confirmed in all strains regardless of the induction levels measured. The observed results confirm findings from other studies that phage induction results in free phage particles, disseminating *stx* genes to the surrounding environment either inside the host intestine or outside when it secreted in host faecal materials (Martinez-Castillo et al., 2013, Tyler et al., 2013).

The role of bacteriophage in the evolution of bacterial strains by horizontal gene transfer has been well documented *in vitro* and *in vivo* (Toth et al., 2003, Tozzoli et al., 2014). The use of the spot agar test in this study demonstrated that Stx1-encoding phage had differences in host range but even when susceptibility was observed, isolation of stable lysogens using this method was not possible. While the presented finding is in agreement with the published reports that Stx1-encoding bacteriophage transduction may occur but a stable lysogeny is rare (Tozzoli et al., 2014, Bonanno et al., 2016), it is also possible that the method used for detection of lysogens resulted in underestimation of lysogens. In fact, when the HGMF method was used to detect presumptive lysogens, more stable lysogens where detected indicating that HGMF is a useful technique for the isolation of lysogens particularly if low numbers of EHEC positive colonies are expected in complex background (Szabo R.A. et al., 1986). The importance of unstable transductants for clinicians and food industry needs to be further understood as their ability to acquire Stx-encoding bacteriophage may be of significance in some clinical and food testing scenarios.

Although more stable lysogens were detected using HGMF, they were in the K12 laboratory strains and generating of lysogens in *stx*-negative strains particularly pEHEC was not possible despite multiple attempts. The SBIs in *stx*-negative O26 strains were vacant suggesting that accessing integration site did not prevent the phage from lysogenising *stx*-

negative O26 strains. In fact, it has been demonstrated that *in vitro* conditions are less effective in transduction of Stx-encoding and lysogenisation of wild-type *E. coli* (Toth et al., 2003). Generation of unstable lysogens could be another factor that limits the detection of lysogens (Sekse et al., 2008, Tozzoli et al., 2014). In this scenario phage integration may have occurred transiently and therefore isolation is problematic as was demonstrated in this study for some strains. Finally, the genetic background of the infective phage that facilitate Stx transduction into a susceptible host could be another favouring perquisite for the transduction to occur (Sekse et al., 2008b).

Quantification of *stx* expression levels by dPCR demonstrated that *stx* expression levels (copy number μl^{-1}) were higher in MMC samples than NMMC either after 3 hr or 6 hr induction. This result further supports that MMC has a detrimental effect on bacterial cells as it results in phage induction, cell lysis and release of Stx-bacteriophage and subsequently Stx toxin (Tyler et al., 2013). The finding also confirms the presence of a heterogeneity in phage induction within EHEC populations in which strains with high spontaneous induction rates would have higher cell lysis and lower persistence rates allowing release of infective phage particles, Stx toxin production and release (Louise and Obrig, 1992, Iversen et al., 2015) which causes damage of the colon blood vessels and occurrence of bloody diarrhoea that may proceed to HC or HUS (Bekassy et al., 2011). Unlike populations with lower spontaneous induction rates would have less cell lysis and higher persistence rates which is important for maintenance of EHEC population inside and outside the host intestine (Los et al., 2012).

The amount of Stx produced by EHEC has been suggested to influence the severity of the clinical outcome with high Stx being produced by EHEC strains isolated from human suffering severe disease (Tyler et al., 2013, Ritchie et al., 2003). In this chapter, cattle and

clinical EHEC O26 strains along with their K12 lysogens were tested by ELISA for their ability to produce Stx. Overall cattle strains in this study produced Stx1 toxin at levels comparable to that of clinical strains, confirming the pathogenic potential of EHEC cattle strains (Mellor et al., 2015). It was also demonstrated that lysogens produced greater quantities of Stx compared to their parent strains. This result is consistent with other studies and provides evidence that lysogenic conversion can contribute to bacterial pathogenesis by generating toxin-producing bacteria (Bielaszewska et al., 2007, Acheson et al., 1998). The increase in Stx1 production by K12 lysogens could also be driven by the regulatory influence of prophage induction. Such a process could enhance Stx production by controlling *stx1* transcription, amplifying *stx1* copy number and/or allowing Stx release by phage-mediated lysis mechanism (Wagner et al., 2002).

4.6 **Conclusion**

In conclusion, this study characterised the insertion loci of Stx1-encoding phage of EHEC O26 derived from clinical and cattle strains in Australia and explored whether the Stx phage was able to transduce *stx*-negative strains, some of which were closely related to EHEC but lack *stx*. Work presented here demonstrated that Stx prophage of human and cattle EHEC O26 strains and corresponding lysogens were predominantly found to be inserted in a novel loci adjacent to *torS*; however the *stx1* prophage boundaries remain undetermined. Evaluating Stx1-encoding phage induction level, production and expression suggested heterogeneity of Stx bacteriophage of *E. coli* O26 strains isolated in Australia. Data presented demonstrated that while induction occurred at higher rates when strains were exposed to inducing agents such as MMC, spontaneous induction was observed at different efficiencies but at lower levels than MMC induction, probably reducing the lytic cycle rate and allowing host survival.

However, spontaneous induction was not predictive of a strain response to mitomycin C induction, toxin production or expression. Additionally, *stx* expression and Stx production were comparable between EHEC strains originating from clinical and cattle sources thereby confirming the pathogenic potential of EHEC cattle strains. Accordingly, the focus on EHEC and associated pathotypes in their reservoir and surrounding environment as a source of EHEC is essential.

Supplementary information to Chapter 4

Data presented here is part of Chapter 4 but not presented within the prepared manuscript

4.7 Materials and Methods

4.7.1 *In silico* PCR for prevalence of phage integrases in *E. coli* O26 genome

Integrase sequences from enteric phages were detected by virtual PCR (Geneious, 9.1.5) (Kearse et al., 2012) using Balding et al., (2005) primers outlined in Table 4.3. Attempts were then made to determine the insertion loci for lambda integrase and subsequently Stx phage. Reference strains outlined in Table 4.3 were used to predict the PCR product size for all integrases.

Table 4-3. PCR primers designed to detect integrase sequences from temperate enteric phages

Integrase	Phage	Organism	Accession No	primers	Product	Reference
Group1	H19J HK022 Lambda P434	*Escherichia coli* O26:H – strain H19 *Escherichia coli* *Escherichia coli* *Escherichia coli*	AJ236875 M60848 J02459 AF069308	F-GTTACMGGGCARMGAGTHGG R- ATGCCCGAGAAGAYGTTGAGC	378	(Balding et al., 2005)
Group 2	e14 P21	*Escherichia coli* K-12 *Escherichia coli*	U00096 M61865	F-GTTACTGGWCARCGKTTAGG R- GATCATCATKRTAWCGRTCGGT	433	(Balding et al., 2005)
Group 3	ST64B	*Salmonella enterica, Typhimurium*	AY055382	F- TGGARATWKCYTATYTVTGTGC R- TCRTARTCTGARATYCCYTTBGC	333	(Balding et al., 2005)
Group 4	933W	*Escherichia coli O157:H7*	AF125520	F-CCTGGTCACTTTGGGGCACGT R-GCTCCATCGGAGCGACATGG	267	(Johansen et al., 2001)
	EH297 Fels-1 Gifsy-2 P27 phi4795	*Escherichia coli O157:H7* *Salmonella enterica,Typhimurium* *Salmonella enterica,Typhimurium* *Escherichia coli* *Escherichia coli*	AJ431361 NC 003197 AE008743 AJ298298 AJ556162	F- CTBGCMTGGGARGATATHGA R- GMCCAGCABGCATARGTRTG	392	(Balding et al., 2005)
Group 5A	HK620 P4 Sf6	*Escherichia coli* *Escherichia coli* *Shigella flexneri*	AF335538 X05947 X59553	F- TGGRAKRAMKTCGAYTTYGA R- AGTTGCMYYTCWATMGCGTCA	360	(Balding et al., 2005)
Group 5B	P4 epsilon15	*Escherichia coli* *Salmonella enterica*	X05947 AY150271	F- TWGTKCGTWMMAGTGAATT R- TKGWTRTATRCCGCWCGYAC	433	(Balding et al., 2005)
Group 5C	Gifsy-1 phi80	*Salmonella enterica, Typhimurium* *Escherichia coli*	AE008819 X04051	F- GGRMARTYATAAAACKSG R- TGCCCGAGCAKCWTYTCA	297	(Balding et al., 2005)
Group 6A	L-413C P2 WPhi	*Yersinia pestis* *Escherichia coli* *Escherichia coli*	NC 004745 AF063097 AJ245959	F- CTGAGYACWGGAGSAMGWTGG R- CCBCCRTTMATCATRAARTG	287	(Balding et al., 2005)
Group 6B	Fels-2 P186 PSP3	*Salmonella enterica, Typhimurium* *Escherichia coli* *Salmonella*	AE008824 U32222 AY135486	F- TVGCWACYGGCGCMMGRTGG R- CCBCCRTTMATCATRAARTG	283	(Balding et al., 2005)
Group 7	P22 SfII SfV ST64T	*Salmonella enterica, Typhimurium* *Shigella flexneri* *Shigella flexneri* *Salmonella enterica, Typhimurium*	AF217253 AF021347 U82619 AY052766	F-ACATYATMAAYCTKGARTGGCA R-CGAACCATTTCKATRGACTCCCA	391	(Balding et al., 2005)
Group 8	P1	*Escherichia coli*	X03453	F-GCTTATAACACCCTGTTACGTAT R- CAGCCACCAGCTTGCATGATC	447	(Balding et al., 2005)

Degenerate Primers code B=(C,G,T), D=(A,G,T), H=(A,C,T), K=(G,T), M=(A,C), N=(A,C,G,T), R=(A,G), S=(G,C), V=(A,C,G), W=(A,T), Y=(C,T), e14; is a defective element, ST64B; inducible but defective phage 933W also represents VT2-Sa. (Table adapted from Balding *et al* , 2005) (Balding et al., 2005).

4.8 **Results**

4.8.1 Prevalence of phage integrases in *E. coli* O26 genome.

Balding *et al*., (2005) classified phage integrases from enteric bacteria into groups based on integrase sequence. The prevalence of phage integrases in the genome of *E. coli* O26 strains is displayed in Figure 4.7. On average each strain was shown to contain at least three integrases. Phage integrase group 5A was shown to be present in all strains examined. Group 5A includes integrases for HK620, P4 from *E. coli* and SF6 from *S. flexneri*. Group 1 lambda integrases were identified in all strains except NTEC while group 7 and group 3 also present at prevalence >77%. Other phage groups were identified at frequencies <20% with groups 2, 5B and 5C not identified in any strain.

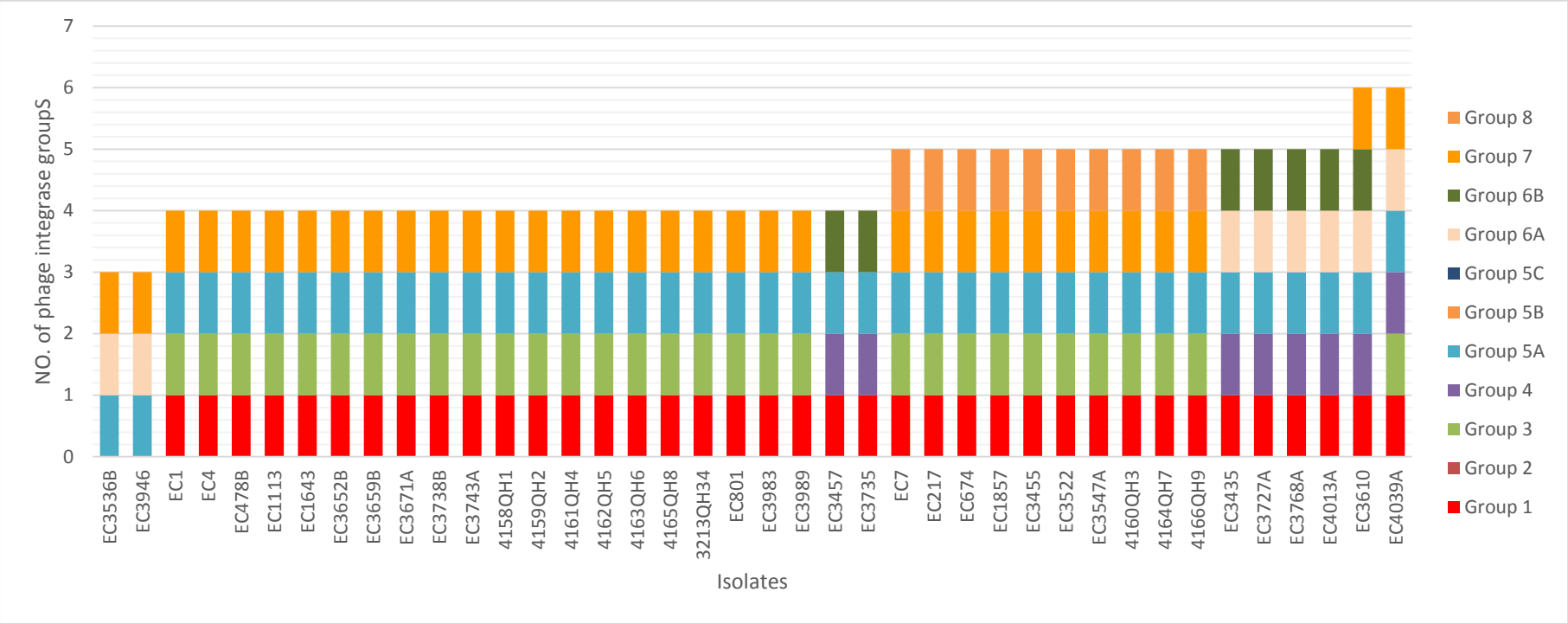


Figure 4-7. Number of phage integrases in each *E. coli* O26 strain.

The association of pathotypes with phage integrase carriage was examined and some relationships identified (Figure 4.8). Group 3 integrases were only found in all EHEC and pEHEC and a single aEPEC (EC4039). Phage integrase group 4 was carried only by aEPEC strains (100%) and phage 6B was found in the majority (87.5%) of aEPEC strains. No difference between clinical and cattle strains in their carriage rate for phage integrases of enteric bacteria was found.

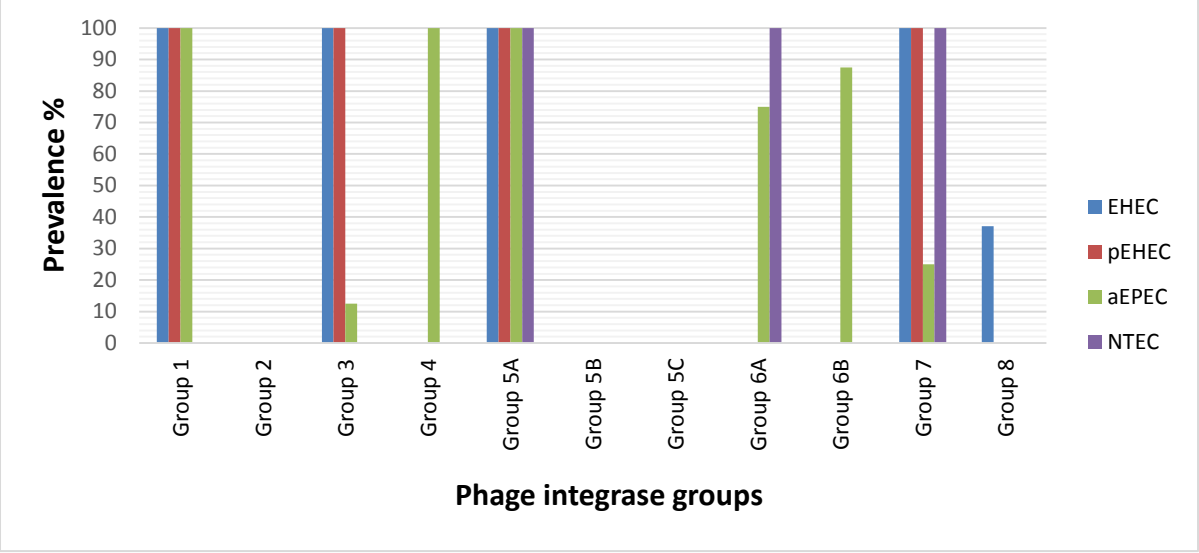


Figure 4-8. Prevalence of phage integrases in *E. coli* O26 pathotypes.

In an attempt to identify Stx phage integrase, degenerate primers for lambda integrases targeting conserved sequences of Box I and Box II in tyrosine recombinases were utilised. A number of lambda integrases were identified bioinformatically with one of them was strongly associated with EHEC. Genes adjacent to the integrases were identified using Blast analyses (National Centre for Biotechnology Information, NCBI, Bethesda, USA). Three lambda integrases were

identified in all EHEC strains except EC4166QH9 which had two lambda integrases, two in pEHEC strains and two aEPEC strains except EC4013 which had only one phage lambda integrases. NTEC strains appeared to not harbour any lambda integrase. The three genes located next to the lambda integrase were *yfaA* (conserved predicted protein), *ybhC (*predicted pectin esterase) and *torS* (sensor protein). The integrase adjacent to *yfaA* was found in all aEPEC, pEHEC and EHEC. The integrase next to *ybhC* was found in seven aEPEC, all pEHEC and the integrase adjacent to *torS* were found only in all EHEC except EC4166QH9. In summary, it is likely that the integrase found in close proximity to *torS* represents lambda bacteriophage that harbour *stx*, and that the remaining integrases identified are unlikely to be associated with *stx* carriage as they are present in many strains that do not harbour Stx.

4.9 **Discussion**

Bacteriophages play an essential role in bacterial evolution via horizontal transfer of virulence factors. Phage diversity can be acquired within bacterial hosts via recombination or substitution events when bacteriophage are exposed to the genetic content of other phages (For review refer to Kruger and Lucchesi (2015)). Heterogeneity of bacteriophage has been demonstrated previously (Bonanno et al., 2016, Fogg et al., 2007, Muniesa et al., 2003) even when bacteriophage has been isolated from a single clone. Results presented here further our understanding of the role bacteriophages play in generating diversity within bacterial populations. Indeed a range of pathotype specific examples were identified during this study. For example, aEPEC could be split into two distinct groups with two strains harbouring group 7 phage integrases or lacking group 6A. Further divergence from aEPEC was evident for aEPEC

EC4039 which clustered with EHEC via its harbouring of ST64B, a lambdoid phage found in *S. Typhimurium* (Tucker and Heuzenroeder, 2004). The aEPEC strain and pEHEC strains clustered with EHEC by harbouring group 3 phage integrases were shown to cluster with EHEC via pulse field gel electrophoresis (PFGE) in Chapter 2.

4.10 **Conclusion**

Variability observed within *E. coli* O26 pathotypes and between *E. coli* O26 strains could be driven by integration of phages into O26 bacterial genome.

Chapter 5

Comparative Genomic Analysis of *E. coli* O26 Strains Isolated from Cattle and Clinical Sources in Australia

5.1 **Abstract**

Background: Between 2001 and 2009, EHEC O26 was found to be associated with 11.1% of all notified EHEC infections in Australia. Cattle are known as a reservoir of EHEC worldwide, however little is known about the relationship between O26 strains from cattle and clinical sources in Australia. This study reports on the use of whole genome sequencing (WGS) and subsequent analysis of 40 *E. coli* O26 strains derived from clinical cases and cattle in Australia for virulence and plasmid profiles. In addition, their clonal and phylogenetic relationships are also described

Results: *E. coli* O26 pathotypes belong to enterohaemorrhagic *E. coli* (EHEC, n=27), potential EHEC (pEHEC, n=3), atypical enteropathogenic *E. coli* (aEPEC, n=8) and non-toxigenic *E. coli* (NTEC, n=2). All *E. coli* O26 pathotypes are H11 except NTEC strains which express H32. *In silico* analysis of genetic markers and MLST demonstrated that the majority of cattle and clinical EHEC O26 belong to ST21, harbour *stx1a, eae*, *espK*, *Z2099, ureD, ureC, Z2098, ehx, katP* and *espP*. The relationship between clinical and cattle strains was further confirmed and general agreement between different *in silico* typing schemes (MLVA, pangenome analysis and LEE- and WG-SNP analysis) was obtained; indicating the pathogenic potential of EHEC O26 cattle strains. pEHEC of O26 although lacking *stx*, appear to contain the necessary attributes of EHEC strains. This group exhibited EHEC virulence determinants, plasmid profiles and clustered with EHEC by MLST (ST21), MLVA, pangenome analysis and SNPs. In addition, the combination of genetic markers including *espK, ureC, ureD, z2098* and *z2099* facilitates the identification of EHEC-like strains that appear to differ from EHEC by the lack of *stx*. This study also

demonstrated that the majority aEPEC are divergent from other pathotypes based on sequence type (ST29) and virulence as well as plasmid profiles. Finally, NTEC had distinct MLST (ST10), MLVA, PCA of pangenome and SNP, exhibited absence of EHEC virulence determinants and all EHEC plasmid profiles.

Conclusion: The use of WGS provides evidence of the continuous evolution and plasticity of the genome of this organism and its ability to undergo genetic rearrangements and further confirms that that there are strong relationships between O26 strains from cattle and humans. Overall, while there appears to be overlap between clinical and cattle strains, all of the EHEC O26 included in this study are *stx1* and are therefore likely to be less virulent/ pathogenic than the recently described *stx2* clones that have appeared across Europe (ST29/ *stx2a/ ehxA+/ katP- / espP- /etpD+) or (ST29/ stx2d/ ehxA-/ katP-/ espP-/ etpD+).*

5.2 Introduction

E. coli O26 is the second most frequently detected enterohaemorrhagic *E. coli* (EHEC) worldwide capable of causing disease in humans ranging from mild diarrhoea to more severe outcomes such as haemolytic uremic syndrome (HUS). Beef cattle and dairy products have been identified as a source of EHEC O26 (Vally et al., 2012, USDA and FSIS, 2012, Severi et al., 2016, Madic et al., 2011, Buvens et al., 2011, Allerberger et al., 2003). Furthermore, this pathogen and other non-O157 including O45, O103, O111, O121, O145 are adulterants of certain raw beef products (USDA and FSIS, 2012). Although the prevalence of *E. coli* O26 in Australian cattle and the burden of EHEC O26 illness on the Australian public health system would appear to be low when compared to other countries (Mellor et al., 2016, Vally et al., 2012, Barlow and Mellor, 2010), detection of this organism is of concern to public health authorities and is of regulatory importance to red meat producers and exporters.

E. coli O26 can belong to a range of pathotypes that are characterised by the virulence markers they carry (Chapter 2). Most attention is given to two main pathotypes EHEC and enteropathogenic (EPEC) and their derivatives. EHEC are characterised by the carriage of *stx*1 and/or *stx2*, *eae* (*E. coli* attachment and effacing gene) and *ehx* (enterohaemolysin) and are the main pathotype of concern due to its involvement in severe foodborne outbreaks. In contrast, EPEC strains lack *stx* but carry *eae* and cause mild diarrhoea (Nguyen et al., 2006). The EHEC pathotype has been further subdivided into EHEC-like O26 or potential EHEC (pEHEC) O26. These derivatives carry many EHEC virulence markers but don't produce Stx. On the other hand, EPEC can be subdivided into typical EPEC (tEPEC) and atypical EPEC (aEPEC). tEPEC strains

are classified based on the presence of EPEC adherence factor (pEAF) plasmid encoding bundle forming pili (*bfp*) (Piazza et al., 2013). In contrast, aEPEC strains lack the EAF plasmid and therefore do not produce *bfp* (Leomil et al., 2005).

Following the emergence of a new O26 clone in Europe (Bielaszewska et al., 2013) several studies were conducted to further characterise *E. coli* O26 populations. The use of next-generation sequencing (NGS) provides evidence of the continuous evolution and plasticity of the genome of this organism and its ability to undergo genetic rearrangements. Indeed it enables the relatedness of EHEC and *stx*-negative *E. coli* O26 to be determined (Gonzalez-Escalona and Toro, 2016, Norman et al., 2015). Despite EHEC being of economic importance to the Australian cattle industry, studies of Australian E. coli O26 isolates to identify their population structure, genetic characteristics and relationship between isolates are yet to be conducted. This study reports on the use of whole genome sequencing (WGS) and subsequent indepth analysis of *E. coli* O26 strains derived from clinical cases and cattle in Australia for antimicrobial resistance genes (AMR), virulence and plasmid profiles. In addition, their clonal and phylogenetic relationships are also described.

5.3 Materials and Methods

5.3.1 Bacterial strains

A total of 40 *E. coli* O26 strains (Chapter 2 and Chapter 3; Table 3.1), representative of diversity in PFGE, sources and years (Chapter 2) observed across the initial set of 88 isolates, were used in

this study. Strains belonged to four pathotypes comprising EHEC, aEPEC, non-toxigenic *E. coli* (NTEC) and pEHEC (Table 5.1).

Table 5-1. Virulence profile of *E. coli* O26 used in the study

Pathotypes	Virulence makers	No of isolates	Source
EHEC	*stx* (*stx*$_1$)$^+$, *eae*$^+$, *ehx*$^+$, *ecf* $^+$, SNP within *rml*A$^+$, *bfp*$^-$	27	Clinical (n=10) and cattle (n=17)
pEHEC	*stx* $^-$, *eae*$^+$, *ehx*$^+$, *ecf* $^+$, SNP within *rml*A$^+$- *bfp*$^-$	3	Cattle
aEPEC	*stx* $^-$, *eae*$^+$, *ehx*$^-$, *ecf*$^-$, SNP within *rml*A$^-$, *bfp*$^-$	8	Cattle
NTEC	Negative for all virulence markers tested	2	Cattle

5.3.2 DNA preparation for sequencing

A colony from each strain was inoculated into 5 ml Luria Bertani (LB) broth and incubated overnight. Genomic DNA was purified from the broth cultures using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions for gram-negative bacteria. Each sequencing mix contained Genomic DNA 1-33.3 as measured by Qubit 2.0 fluorometer (Invitrogen, Life Technologies) using the Qubit dsDNA (double-stranded DNA) HS (High sensitivity) assay kit (Invitrogen) following the manufacturer's instruction. The resultant DNA extract was stored at -20 °C until it was submitted for sequencing.

5.3.3 Sequencing, contigs assembly and annotation

DNA quality control, library preparation and sequencing runs were performed at the Ramciotti Centre for Genomics, University of New South Wales. The Nextera XT DNA library preparation kit (Illumina) were used for DNA library construction and the MiSeq desktop sequencer v3 (Illumina) was used for generation of paired-end (2x300bp) reads (short reads). The fastq-mcf program, v.1.04.676 (https://code.google.com/archive/p/ea-utils) were utilised for Illumina MiSeq reads quality filtering, adapters removing, and clipping. The idba-ud v.1.1.2 program (https://github.com/loneknightpy/idba) with kmers from 150 to 300 bp in length in 50 bp steps was used to perform the reads assembly. Contigs shorter than 1 kb in length were removed from the overall analysis to increase reliability.

5.3.4 Comparative genome fingerprinting (CGF)

WGS data were analysed using Centre for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/) web-based tools. SeroTypeFinder (Joensen et al., 2015) (https://cge.cbs.dtu.dk/services/SerotypeFinder/) was utilised to confirm the strains serotype, VirulenceFinder database (https://cge.cbs.dtu.dk/services/VirulenceFinder/) (Joensen et al., 2014) was used for detection of virulence genes (Appendix 1), with the threshold for ID was set at 95% over 80% of the gene's length. Assembled genomes or MiSeq reads were submitted for online identification and a local version of the blast procedure for identification was also performed using a python script (https://github.com/tallnuttcsiro/pathtype.py) with a local copy of the virulence gene database. In addition, the distribution of virulence genes on pathogenicity islands (PAIs) or throughout the genome that are not included in the CGE virulence gene

database were examined by *in silico* PCR using primers shown in Table 5.2. In addition, the *arcA* type and SNPs within O antigen gene cluster sequences (*rmlA*, *wzx* and *fnl1)* for *E. coli* O26 strains was extracted and aligned with corresponding sequences deposited to GenBank; accession no. AJ875429 (EHEC O26, *arcA2*), AJ875430 (EPEC O26, *arcA2*) (Leomil et al., 2005) and AY763106 (*rmlA*, *wzx* and *fnl1*). Identification of plasmids were also performed using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) with the selected database Enterobacteriaceae, the threshold for ID was set at 95% and assembled genome contigs was set as the read type. For assigning strains to multi locus sequence typing (MLST), MLST database (https://cge.cbs.dtu.dk/services/pMLST/) was used, *E. coli* scheme 1 was selected for MLST configuration and assembled genome contigs was set as the read type.

5.3.5 *In Silico* detection of antimicrobial resistance (AMR) genes

ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) was used to determine the presence of AMR genes with the following configuration set used for all antimicrobials: the threshold for the ID was 95%, 80% for the length, and assembled genome contigs was set as the read type. The AMR genotype was compared to the previously determined AMR phenotype (Chapter 2), and any differences were further examined by BLAST searching for detection of specific AMR genes within genome assemblies.

Table 5-2. Gene targets and PCR primers for the detection of additional virulence markers by *in silico* PCR

Target	Location	Function	Primers	Product Size	References
adfO	OI-57	Adhesin	F-TGGTGGCCCGCATACAGC R: TGCCCAGTCAGCCCAGGTTA	501	(Imamovic et al., 2010)
chuA	Chromosome	Haem/haemoglobin receptor	F-GACGAACCAACGGTCAGGAT R-TGCCGCCAGTACCAAAGACA	279	(Clermont et al., 2000)
ckf	OI-57	Putative killer protein	F-ATGCTCGTCACATATAGATTG R- GTTCGTAAGCTGTGAAGACA	201	(Imamovic et al., 2010)
ent/espL2	OI-122	Microcolony formation and F-actin aggregation	F-GAATAACAATCACTCCTCACC R-TTACAGTGCCCGATTACG	233	(Coombes et al., 2008)
nleA	OI-71	Disruption tight junctions and protein trafficking	F-ATGAACATTCAACCGACCATAC R- ACTCTTGTTTCTTGGATTATATCAAA	1296	(Coombes et al., 2008)
nleB2	OI-36	Non-LEE encoded type III effector	F-GTTAATACTAAGCAGCATCC R- CCATATCAAGATAGATACACC	475	(Coombes et al., 2008)
nleD	OI-36	Immunomodulation, zinc-metalloprotease	F-GGTATTACATCAGTCATCAAGG R- TTGTGGAAAACATGGAGC	426	(Coombes et al., 2008)
nleE	OI-122	Immunomodulation	F-GTATAACCAGAGGAGTAGC R- GATCTTACAACAAATGTCC	260	(Coombes et al., 2008)
nleF	OI-71	Disruption protein trafficking	F-ATGTTACCAACAAGTGGTTCTTC R- ATCCACATTGTAAAGATCCTTTGTT	567	(Coombes et al., 2008)
nleG	OI-71	Ubiquitin ligase	F-ATGTTATCGCCCTCTTCTATAAAT R- ACTTAATACTACACTAATAAGATCCA	902	(Coombes et al., 2008)
nleG2–1	OI-71	Ubiquitin ligase	F-ACCAGAAACCTGACTTCG R-CAGCATCTTCATATACTACAGC	406	(Coombes et al., 2008)
nleG2–3	OI-57	Ubiquitin ligase	F-GGATGGAACCATACCTGG R-CGCAATCAATTGCTAATGC	551	(Coombes et al., 2008)
nleG5–2	OI-57	Ubiquitin ligase	F-TGGAGGCTTTACGTCATGTCG R-CCGGAACAAAGGGTTCACG	504	(Coombes et al., 2008)
nleG6–2	OI-57	Ubiquitin ligase	F-CGGGTCAGTGGATGATATGAGC R-AAGTAGCATCTAGCGGTCGAGG	424	(Coombes et al., 2008)
nleG9	OI-71	Ubiquitin ligase	F-GTTCGTGCCCGAATTGTAGC R: CACCAACCAAACGAGAAAATG	409	(Coombes et al., 2008)
nleH1–1	OI-36	Immunomodulation	F-GTTACCACCTTAAGTATCC R-GTTTCTCATGAACACTCC	456	(Coombes et al., 2008)
nleH1–2	OI-71	Immunomodulation	F-AACGCCTTATATTTTACC R-AGCACAATTATCTCTTCC	589	(Coombes et al., 2008)
terB	OI-43/ OI-48	Tellurite resistance B	F-GCCAGGTTGGCCGTTTC R-CCGTCACTCGATACGGCAAT	82	(Tzschoppe et al., 2012)
TspE4.C2a	Chromosome	Esterase-lipase protein	F-GAGTAATGTCGGGGCATTCA R-CGCGCCAACAAAGTATTACG	152	(Clermont et al., 2000)
ureC	OI-43/ OI-48	Urease-associated protein C	F-TCTAACGCCACAACCTGTAC R-GAGGAAGGCAGAATATTGGG	397	(Nakano et al., 2001)
yjaA	Chromosome	Unknown	F-TGAAGTGTCAGGAGACGCTG R-ATGGAGAATGCGTTCCTCAAC	211	(Clermont et al., 2000)
espV	OI-44	Secretion	F-TCAGGTTCCTCGTCTGATGCCGC R-CTGGTTCAGGCCTGGAGCAGTCC	87	(Delannoy et al., 2013a)
espK	OI-50, Prophage CP-933N	Secretion	F-GCAGRCATCAAAAGCGAAATCACACC R-TCGTTTGGTAACTGTGGCAGATACTC	110	(Delannoy et al., 2013a)
Z2099	OI-57	Unknown	F-TAGCGGGACAATTGTCACGG R-GTCTTTCGGAGAAACATTCTGCC	67	(Delannoy et al., 2013b)
Z2098	OI-57	Unknown	F-CTGAAAAGAGCCAGAACGTGC R-TGCCTAAGATCATTACCCGGAC	136	Delannoy et al., 2013)

z2121	OI-57	Unknown	F-GATGGCAGATAATAACGAAGCAAC R-CAGCCGTTGAAGCATCAGCG	118	Delannoy et al., 2013)
terC	OI-43/48	Tellurite resistance C	F-TCC TGG CGC TGA AAG AT R-GAA ACA CTC ATA AAA TAACCT CTT	1240	(Taylor et al., 2002)
aid-1	OI-43/48	Adherence Inducing Determinant 1	F-ACTGGTTACCAGTACTGCTG R-ACCAGTCTTCATCGCTGTCA	883	(Nakano et al., 2001)
irp2	HP	Iron repressible protein	F-AAGGATTCGCTGTTACCGGAC R- TCGTCGGGCAGCGTTTCTTCT	280	(Schubert et al., 1998)
fyuA	HP	Pesticin receptor	F-GCGAC GGGAAGCGA TTTA R-CGCAGTAGGCACGATGTTGTA	780	(Schubert et al., 1998)
pagC	OI-122	promote resistance to macrophage digestion	F-ATGAGTGGTTCAAGACTGG R-CCAACTCCAACAGTAAATCC	521	(Karmali et al., 2003)
ureD	OI-43/48	Urease-associated protein D	F-GCAATAATTGACTCTGATTGCC R-GCTGCTGCGGTAAAATTTACT	69	(Delannoy et al., 2013a)

5.3.6 Analysis of pangenome data

The pangenome (i.e. all coding sequences found among all strains) was produced by the following method: 1. coding sequences (CDS) for all strains were identified using Prokka v.1.11 (http://www.vicbioinformatics.com/software.prokka.shtml) in 'fast' mode. 2. All CDS were amalgamated into one multi-fasta file. The Usearch v.8.1.1861 program (http://www.drive5.com/usearch/) cluster fast command was used to find clusters of CDS with a 60% amino acid identity threshold. 3. The Usearch cluster output was parsed into a pangenome CDS table using a python script (https://github.com/tallnuttcsiro/vpcr.py/blob/master/gene-matrix-from-uclust3.py) and the CDS Usearch centroid file was used to obtain annotations for the pangenome table via a Blast search. Pangenome CDS were ranked in order of their high and low frequency in different factors: O26 EHEC vs. aEPEC; pEHEC and NTEC, human vs. cattle; and biofilm and non-biofilm forming strains. These ranks were determined by sorting the table of all accessory CDS (not present in all genomes). Each accessory CDS from all the genomes examined was assigned a pangenome score which was calculated by multiplying the highest

Usearch percentage identity by the percentage query coverage. Once the pangenome was sorted by the desired parameter, (e.g., strain source), the pangenome scores were summed within the two broad groups. The summed values were then subtracted to yield a final score used to rank the proteins. Tables were then sorted by the ranking score to determine the core genes (present in all genomes), the accessory genes (not present in all genomes) and pathotype specific genes (Genes specific for each pathotypes). A python script was used to calculate the PCA pangenome (https://github.com/bioinformatics-deakin/PCA) with the input file was the gene table for all samples.

5.3.7 *In Silico* multi-locus variable number tandem repeat analysis (MLVA)

Primers designed by Lindstedt et al. (2007) and Lobersli et al. (2012) were used to screen assembled genomes and find possible PCR products with the python script, mlva.py (https://github.com/tallnuttcsiro/mlva.py/blob/master/mlva.py). A minimum spanning tree was generated using the following MLVA repeat regions; CVN001, CVN004, CVN007, CVN014, CVN015, CVN016 and CVN017. In order to allow analysis without exclusion of samples with missing values, strains that had undetermined values for CVN016 (n = 5) and CVN017 (n = 6) were assigned a value that was equal to the most frequently observed for each corresponding locus. Repeat values were imported to BioNumerics (version 7.6; Applied Maths, Austin, TX) as character data that were subsequently used to generate a minimum spanning tree (MST).

5.3.8 Whole genome-single nucleotide polymorphisms (WG-SNPs)

SNP analysis was performed comparing O26 strains genome with O26 genome of AP010953 as a reference strain. The program, Parsnp v.1.2 (Rapid core genome multi-alignment) was used to find SNPs among the strains' genome assemblies (https://github.com/marbl/parsnp). Finally, only SNPs which showed a minimum variance of 0.05 among strains were used for principal components analysis (PCA) to reduce the potentially very large number of SNPs to a manageable level, while retaining any structure present in the data.

5.3.9 SNP within locus of enterocyte effacement (LEE)

All filtered and clipped reads were mapped to the O26 LEE reference (AP010953). The mapped reads were then assembled by idba. The LEE assemblies were then blasted against the reference strain to make a circles diagram (https://github.com/bioinformatics-deakin/circles). The annotation on the outer ring marks the end of each gene. The NTEC strains (N=2) had no LEE PI and subsequently they were excluded from the analysis. For SNP analysis, a core SNP set was made using Parsnp (parsnp.vcf, parsnp.mfa) and a dendrogram was generated in BioNumerics, (V. 7.6.1) with simple matching coefficient and unweighted paired-group method (UPGMA) with arithmetic mean values on the basis of presence or absence of targets genes. SNP position within LEE and the name of the gene (e.g. 13149-*escI*) were imported from Excel sheet to BioNumerics and a score of "1" was given to the present SNP and a score of "0" was given to the absent SNP.

5.4 Results

5.4.1 Comparative genome fingerprinting (CGF)

The O:H type for 40 strains previously characterised as O26 by PCR were determined using CGE. The results of the WGS-based *in silico* serotyping showed that 26 (65%) strains were identified as *E. coli* O26. Fourteen (35%) strains (5 clinical and 9 cattle) had unknown O type using *in silico* serotyping due to incomplete assembly of the O antigen clusters in these strains. All strains were H11 except the NTEC which were H32.

Further analysis was conducted to identify the virulence attributes for each strain. A dendrogram was generated to assess the relationship between strains and enable further interrogation of the virulence genes data (Figure 5.1). Among pathotypes, 25 unique virulence profiles were identified in EHEC (n=27 strains), three in pEHEC (n=3 strains), seven in aEPEC (n=8 strains) and one in NTEC (n=2 strains). The number of virulence genes identified ranged from 30 to 43 in EHEC, 31 to 37 in pEHEC, 24 to 39 in aEPEC. Not surprisingly, NTEC harboured the lowest number of virulence genes with only *iss, yjaA* and *gad* detected.

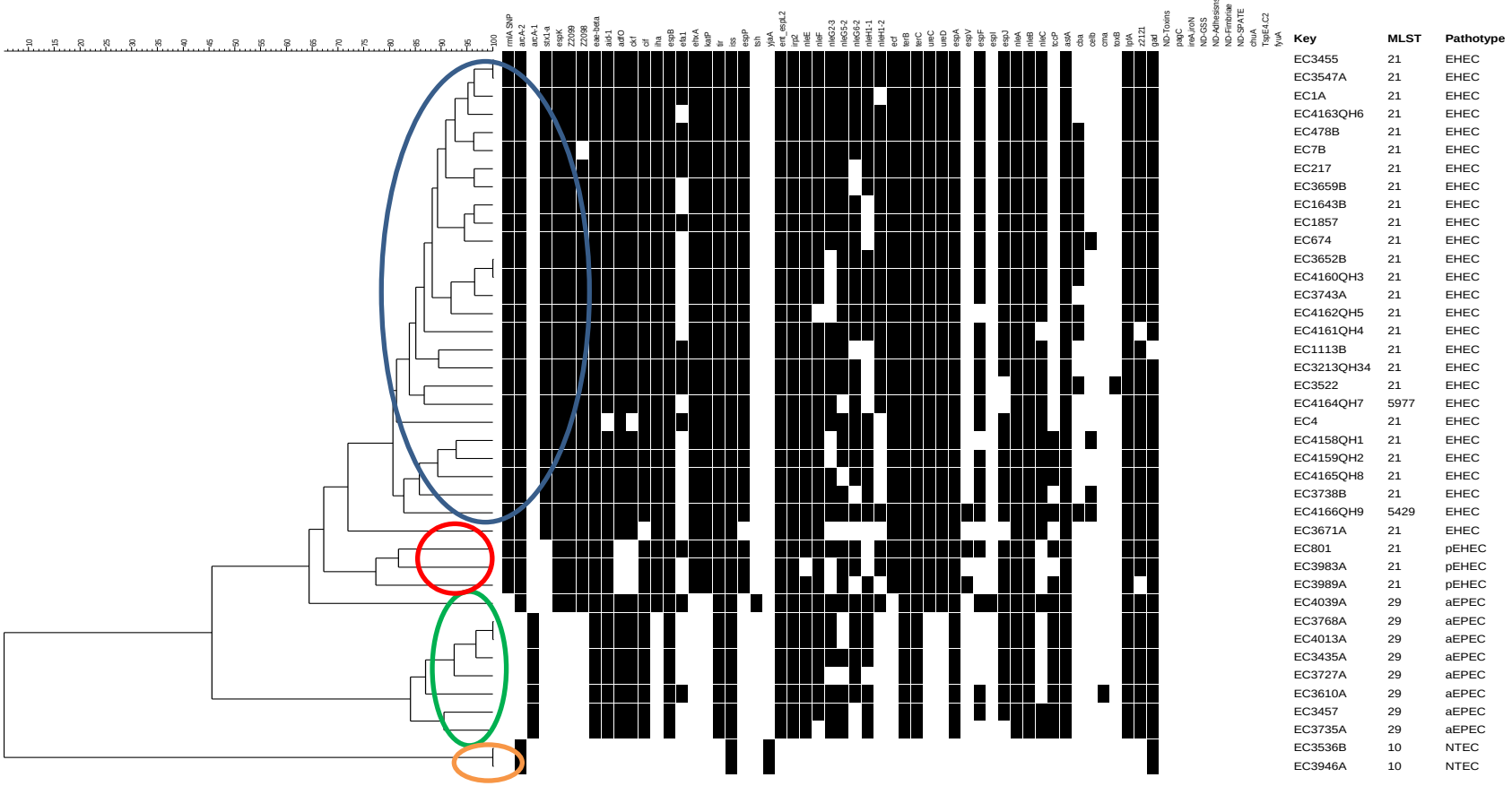


Figure 5-1. **Cluster analysis of virulence genes identified in *E. coli* O26 strains.** Dendrogram was generated using BioNumerics v. 7·6. ND; Not Detected, GSS: Genes encoding Secretion System, ND-Toxins: *mcmA, pet, sat, senB, sta1, stb, virF,ccI,cdtB,cnf1,hlyE, ipaD, ltcA, ltcA, mchB, mchC, mchF*; ND-GSS: *espC, etpD, nleB2, nleD, nleG2-1, nleG9*, ND-Adhesins: *fasA, fedA, fedF, fim41a, nfaE, saa*; ND-Fimbriae: *fanA, K88ab, lngA, perA, prfB, sfaS, bfpA, cfaC, cofA, f17G* and *f17A*, ND-SPATE: *eatA, epeA, pic, repA, sepA, sigA* and *vat.* Strains enclosed within blue oval boundary, red oval boundary, green oval boundary and orange oval boundary are EHEC, pEHEC, aEPEC and NTEC respectively.

The distribution of virulence genes among EHEC, pEHEC, aEPEC and NTEC are shown in Figure 5.2. All EHEC strains were shown to contain *stx1* subtype a. All remaining strains were confirmed as not containing *stx*. With the exception of NTEC strains, all strains belonged to phylogenetic group "A" with the profile: *chuA*$^{-}$, *TspE4.C2*$^{-}$ and *yjaA*$^{-}$. NTEC strains had the profile: *chuA*$^{-}$, *TspE4.C2*$^{-}$ and *yjaA*$^{+}$ (Clermont et al., 2000). The prevalence of genes encoding additional toxins (*virF, toxB, cba, astA, cma* and *celB*) ranged from 2.5-95% across all strains, with *astA* found in 100% of EHEC, pEHEC and aEPEC whereas *cba*, *celb* and *toxB* were detected only in EHEC at 48.15%, 14.8% and 3.7% respectively. Genes involved in resistance and persistence functions located on PAIs OI-43 or OI-48 (*terB*, *terC*, *ureD* and *ureC*) fluctuated between 12.5%-100%. While *terB* and *terC* were detected in 100% of EHEC, pEHEC and aEPEC, differential distribution was observed for *ureD* and *ureC* which were found only in all EHEC and pEHEC but in one aEPEC strain (EC4039A). *ecf* was identified in all EHEC and pEHEC but not in other pathotypes. Of genes encoding for adhesins (*fasA*, *fedA, fim41a*, *nfaE, saa, eae, efa1, espB adfO, aid-1* and *iha*) only *eae, efa1*, *espB, aid-1* and *iha* were detected. Although some genes (*espB*, *eae*, *aid-1*) were found in 96.3-100% of pathotypes (EHEC, pEHEC, aEPEC), differences between the prevalence of *iha* and *adfO* were evident between pathotypes. *iha* was found in all EHEC and pEHEC but in only one aEPEC (12.5%) (EC4039A). In contrast, *adfO* was detected in all EHEC and aEPEC but was absent in pEHEC and NTEC.

The prevalence of genes involved in secretion functions (*ent_espL2*, *espA*, *nleA* and *nleB*) and miscellaneous markers (*tir* and *iss)* were at 100% in pEHEC, EHEC and aEPEC. However, notable differences in the prevalence of other genes encoding for secretion (*tccP*, *espF*, *nleC*, *espK* and *nleH1-2*), miscellaneous markers (*katP* and *ehx*) and unknown function genes carried

on OI-57 (*Z2099* and *Z2098*) were identified between pathotypes (Figure 5.2). For instance, the carriage of *tccP* by aEPEC was significantly (*P* value =0.0001) higher than EHEC but not different from pEHEC. The secreted protein *espK* and the unknown function genes carried on OI-57 (*Z2099* and *Z2098*) were present in EHEC and pEHEC but absent in all aEPEC except one strain (EC4039A). The miscellaneous markers *ehx*, *katP* and the SPATE (*espP*) carried on O26 plasmid were present at 96.3-100% of EHEC and pEHEC but absent in all aEPEC strains. *espF* and *nleC* appeared to be overrepresented in EHEC than pEHEC and aEPEC. In contrast, *ckf* encoding for putative killer protein was identified in 96.3% of EHEC and 100% aEPEC but was absent in pEHEC. Of note, 40% of clinical strains possessed *tccP* gene but it was not found in cattle strains (0/17) whereas *efa1* was overrepresented in EHEC cattle strains (10/17 strains) when compared to human strains (1/10). *arcA2* was found in 100% of EHEC, 100% of pEHEC, 100% of NTEC and a single aEPEC (EC4039A). In contrast, *arcA1* was found 87.5% of aEPEC (87.5%). SNPs within *rmlA* and *fnl1* were identified only in EHEC and pEHEC but not in other pathotypes while identification of SNPs within *wzx* was not possible due to presence of incomplete *wzx* sequences in all assemblies.

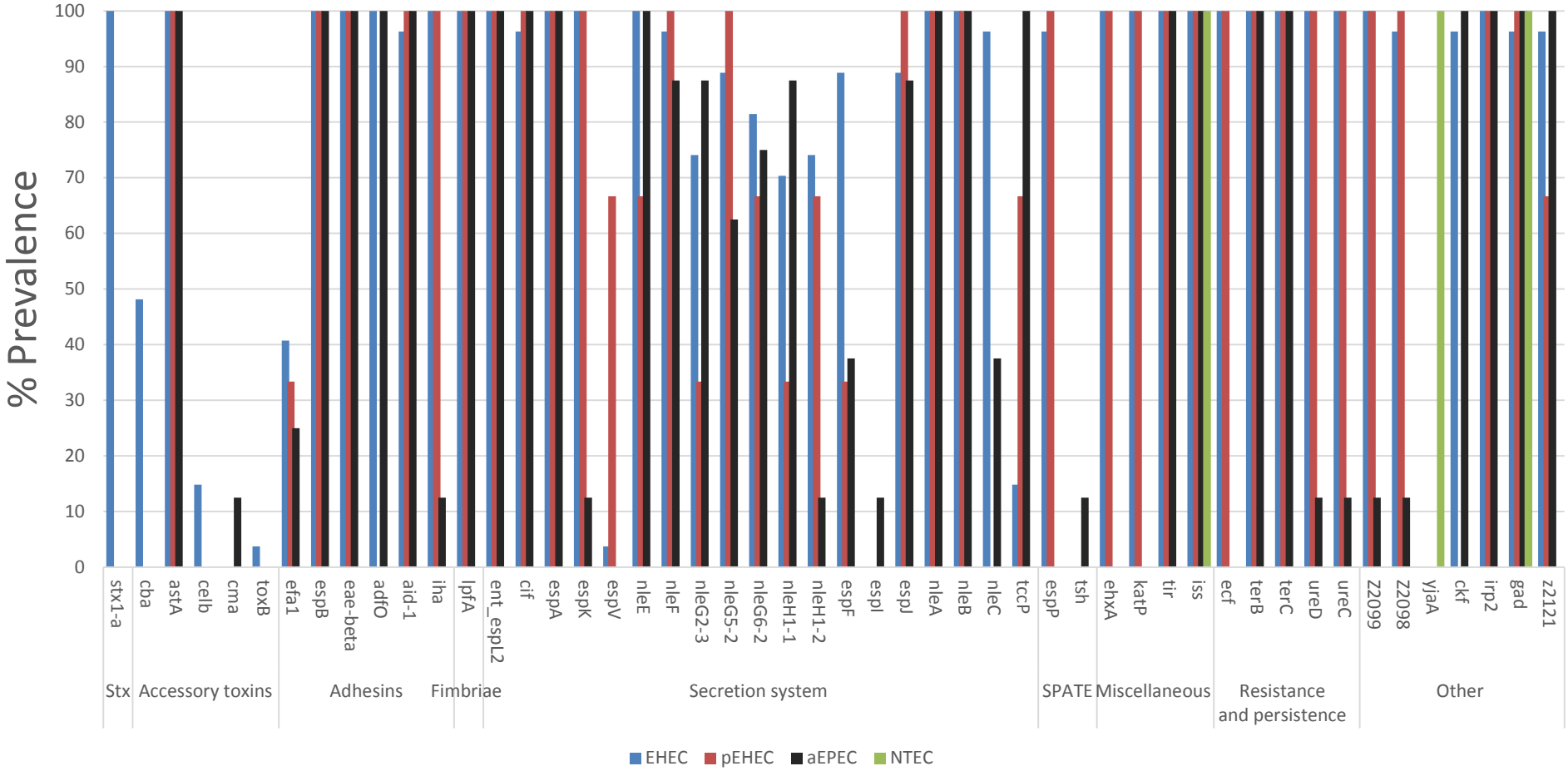


Figure 5-2. Distribution of virulence genes encoding Stx, additional toxins, adhesins, fimbriae, secretion, SPATE, miscellaneous markers, resistance and persistence as well as other functions in *E. coli* O26 from human and cattle sources.

Analysis of the plasmid profiles of Australian *E. coli* O26 strains showed that among 116 specific plasmid replicon sequences deposited in PlasmidFinder database, 17 plasmid replicon sequences were identified in *E. coli* O26 strains included in this study (Figure 5.3). The distribution of these plasmids varied between strains, fluctuating between one and five. For instance, a single strain carried one plasmid, 12 strains carried two plasmids, 19 strains carried three plasmids, seven strains carried four plasmids and a single strain carried five plasmids. Among pathotypes, eight plasmids were identified in EHEC, eight in aEPEC, three in pEHEC and five in NTEC. The prevalence of the 17 plasmids among pathotypes identified some clusters (Figure 5.3). Both IncB/O/K/Z (pO26-CRL) and IncFIB (AP001918) plasmids were predominant among EHEC and pEHEC strains while IncI1 was found in 87.5% of aEPEC and NTEC was characterised by carrying IncFIC (FII) plasmid.

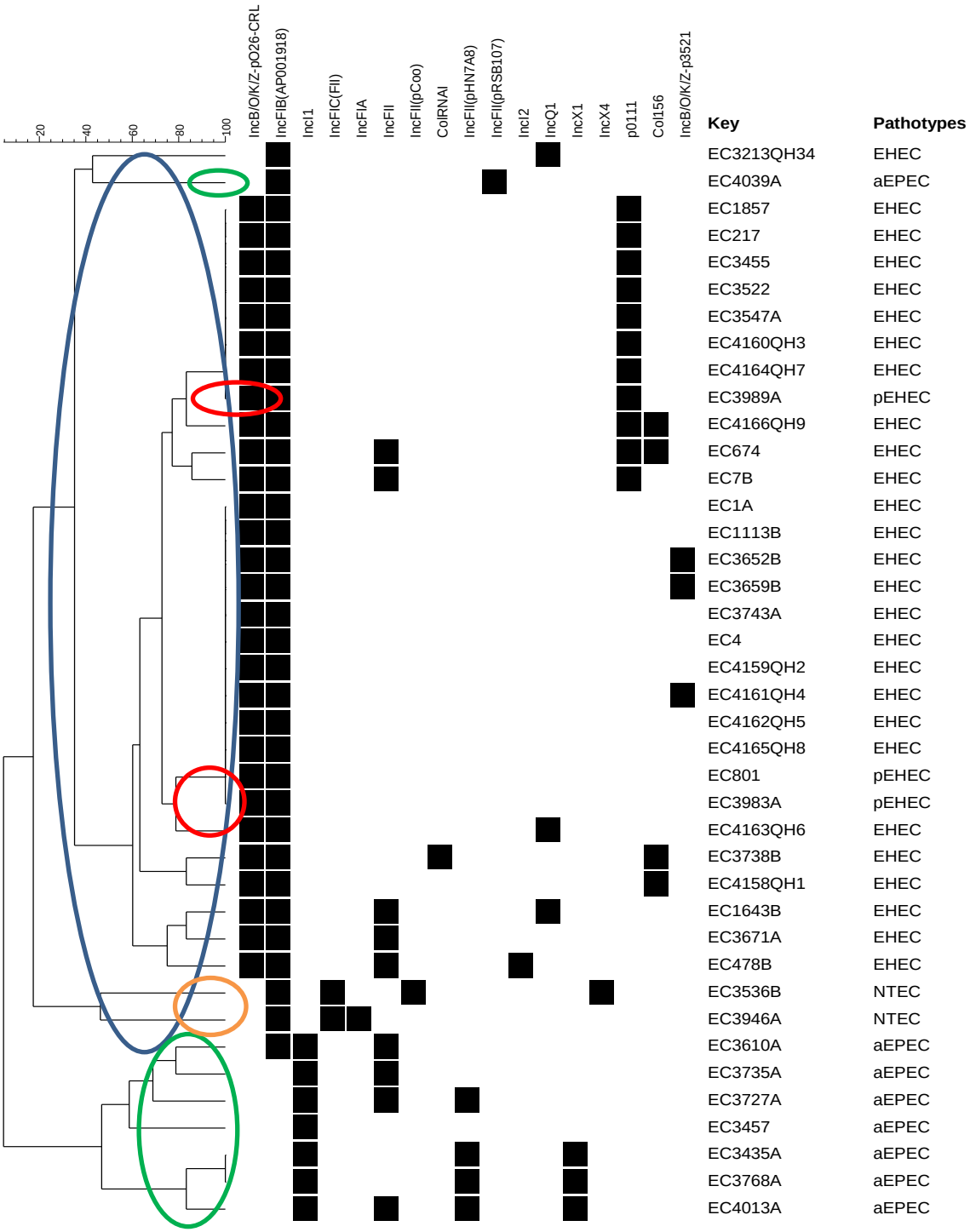


Figure 5-3. Cluster analysis of plasmids identified in *E. coli* O26 strains. Dendrogram was generated using BioNumerics v. 7·6.1. Strains enclosed within blue oval boundary, red oval boundary, green oval boundary and orange oval boundary are EHEC, pEHEC, aEPEC and NTEC respectively.

In addition, MLST analysis revealed that O26:H11/H32 could be clustered into ST based on their pathotypes except EHEC and pEHEC which shared the same ST type. aEPEC strains (n=8) were sequence type 29 (ST29), NTEC strains (two strains) were ST10 and both pEHEC(three strains) and 25 (92.6%) EHEC were ST21. The remaining two EHEC strains that were not ST21 were ST 5977 and ST5429 and were both of human origin.

5.4.2 *In Silico* detection of AMR genes

Antimicrobial resistance genes were found in 11 of the 40 strains; however, genes encoding resistance to antimicrobials of critical importance to human medicine were not observed. Of the total strains positive for AMR genes, two carried a single tetracycline resistance gene (*tetC* or *tetA*), three carried two streptomycin resistance genes (*strA* and *strB*), while the remaining six carried multiple antibiotic resistance genes (Table 5.3). The mechanism for resistance to multiple antibiotics was not fully investigated; however, no linkage to specific mobilizing elements or integrons was detected. Among pathotypes, nine EHEC were found to carry at least two AMR genes whereas *tetA* or *tetC* were detected in a single NTEC and a single aEPEC strain respectively. No AMR genes were detected in pEHEC strains.

Table 5-3. List of strains carried AMR genes and their predicted phenotype

Strains No.	Source	Pathotype	Phenotypic test [a]	Genotype [b]
EC4013A	Cattle	aEPEC	tet	*tetC*
EC3727A	Cattle	aEPEC	amp, str, tet	No resistance genes
EC4163QH6	Clinical	EHEC	amp, kan, str, tet	*strA, strB, aph(3')-Ia,blaTEM-1B, sul2, tetA*
EC4161QH4	Clinical	EHEC	chl, str	*strA, strB, floR, sul2,*
EC3659B	Cattle	EHEC	amp, str	*strA, strB,blaTEM-1C, sul2*
EC3652B	Cattle	EHEC	amp, str	*strA, strB, blaTEM-1C, sul2*
EC3213QH34	Clinical	EHEC	amp, kan, str, tet	*strA, strB, aph(3')-Ia,blaTEM-1B, sul2, tetA*
EC1857	Cattle	EHEC	nal	No resistance genes
EC1643B	Cattle	EHEC	amp, kan, str, tet	*strA, aph(3')-Ia, strB,blaTEM-1B, sul2, tetA*
EC478B	Cattle	EHEC	str	*strA, strB*
EC7B	Cattle	EHEC	str	*strA, strB*
EC674	Cattle	EHEC	Susceptible	strA, strB
EC3946A	Cattle	NTEC	Susceptible	*tetA*

[a] AMR Phenotype: Ampicillin (amp), Kanamycin (kan), Streptomycin (str), Tetracycline (tet), Chloramphenicol (chl), Nalidixic Acid (nal). [b] AMR class detected: Aminoglycoside (*strA*, *strB*, *aph* (3')-Ia), Beta-lactam resistance (*blaTEM*-1B, *blaTEM-1C*), Sulphonamide (*sul2*), Tetracycline (*tetA*, *tetC*), Phenicol (Florfenicol*; floR*).

5.4.3 Analysis of pangenome data

The pangenome of the strains in this study was determined to be 7444 genes. Statistical analysis demonstrated that the average of genome size was 4967.6 +/-172.4 (mean ± standard deviation) and the genome size ranged from 4210.8-5229.3 genes. Comparative pangenome

analysis of *E. coli* strains examined in this study revealed that 49% (3628/7444) of the pan-genome comprised the core genome "genes that are present in all strains". A further 40% (2955/7444) of the pangenome made up the accessory genome "variable genes across groups and present in some but not all strains" and unique genes which they found in only one pathotype. Comparative genome analysis between 40 *E. coli* O26 genome are presented in table 5.4.

Table 5-4. Comparative genome analysis of pangenome, core and accessory genome and unique genes for *E. coli* O26 pathotypes

Pathotype	Pangenome	Core genome	Accessory genome	Strain specific genes within the same pathotype	Unique genes among pathotypes
EHEC	6158	4311	1384	463	310
aEPEC	5849	4237	1080	532	187
pEHEC	5190	4599	187	404	34
NTEC	4855	- [a]	-	-	-

[a] 4114 genes were present in NTEC strains(n=2), 741 gene were present in one strain; however the number of strains is very small to determine the gene category

The pangenome data revealed that the main defining feature of NTEC was the presence of general secretion proteins (type II secretion system) (*gspA*, *gspB*, *gspE*, *gspD*, *gspH*, *gspI*, *gspJ*, *gspK*, *gspL*) and a number of uncharacterised proteins in aEPEC and pEHEC. It is noteworthy that a biofilm development YmgB/AriR family protein was found in all pathotypes but not in aEPEC. PCA analysis of the pangenome revealed that strains of NTEC and 87.5% of aEPEC were segregated into clusters based on their pathotypes whereas EHEC and pEHEC were located together in a single cluster. Of note, aEPEC strain EC4039A that had been shown in Chapter 2 to harbor a range of EHEC virulence markers except *stx* was

shown to be genetically more distant from the rest of the EPEC and continued to demonstrate a strong association with EHEC O26 strains (Figure 5.4A). Finally, there was no clustering of human and cattle EHEC strains based on source thereby demonstrating the relatedness of clinical and cattle strains as well as reinforcing the pathogenic potential of EHEC O26 cattle strains (Figure 5.4B).

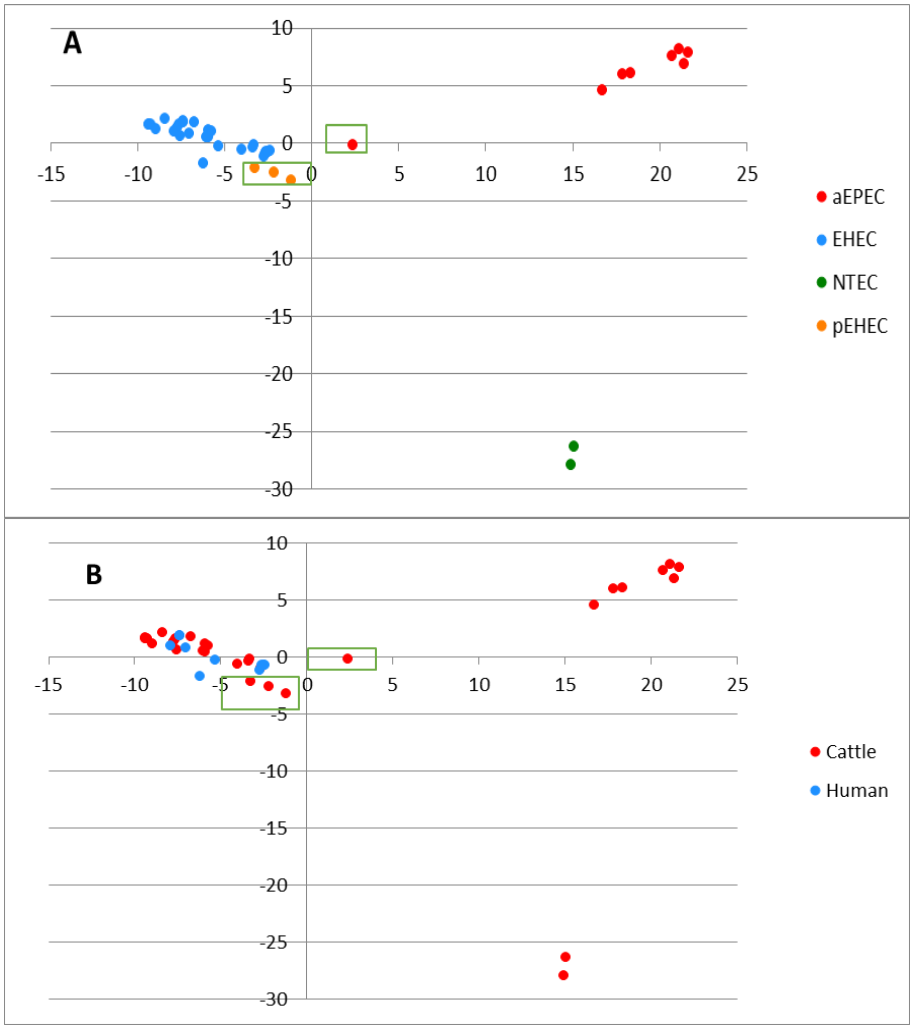


Figure 5-4 A & B. PCA analysis of the pangenome of *E. coli* O26 using (A) pathotype as a designator. B) or source as the designator. The single aEPEC strain (EC4039A) and the three pEHEC O26 strains exhibiting EHEC characteristics are shown within the left hand and are enclosed within a box.

5.4.4 *In Silico* MLVA

To assess the relationship between strains of different pathotypes and different sources a minimum spanning tree (Figure 5.5) was generated. Results demonstrated that 30/40 strains possessed distinct MLVA types. There were seven distinct MLVA types identified from clinical strains (n=10) and 23 from cattle strains (n=27). The remaining ten strains that did not possess unique MLVA profiles and were distributed across five nodes of two strains of the same source except one node which was composed of a clinical and cattle strain (EC3743A and EC4165H8). Overall, isolates separated into four main clusters; NTEC (2/2) and 6/8 aEPEC strains formed two separate clusters while EHEC, pEHEC and 2/8 aEPEC divided between the two remaining clusters (Figure 5.5).

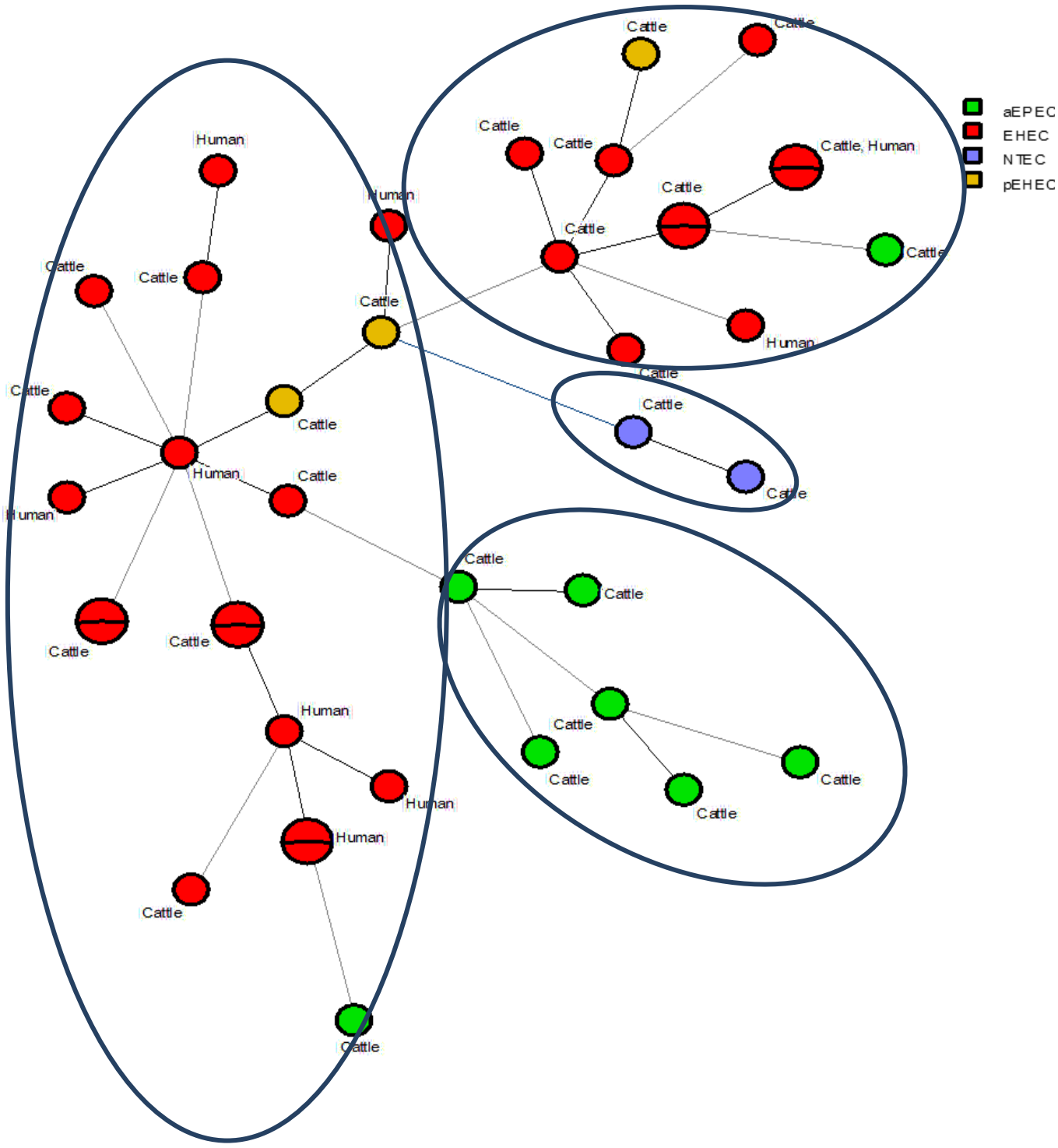


Figure 5-5. A minimum spanning tree for *E. coli* O26 isolates generated using multi-locus variable number-tandem repeats (MLVA). Each node on the tree represents a unique MLVA type while divisions within nodes represent the number of strains that contain the same MLVA profile. Strains are colour coded by pathotypes (EHEC, pEHEC, aEPEC and NTEC).

5.4.5 WG-SNPs

SNP analysis identified 6109.73±10377.6 SNP using O26 reference strain (AP010953) (mean ± standard deviation). The range of the SNPs for all strains was 319-50249. There were 3156.19±1611.4 SNPs in EHEC, 6160.88±559.04 SNPs in aEPEC, 2254±1777.44 SNPs in pEHEC and 50061.5±265.17 in NTEC. PCA analysis of SNPs showed separation based on pathotypes (Figure 5.6A) for some strains. For example, NTEC strains were in a single cluster and seven of eight aEPEC strains clustered together. EHEC separated into two clusters (A and B) with cluster A had 19 EHEC (five human strains and 14 cattle strains) and cluster B were heterogeneous; containing eight EHEC (five human and three cattle), a single aEPEC strain (EC4039A) and three pEHEC strains. Within cluster B (Figure 5.6A) only two EHEC strains (EC4161QH4, EC4166QH9) clustered very closely with aEPEC and pEHEC strains. Clustering based on source (Figure 5.6B) was not evident from the PCA analysis of SNPs as both human and cattle strains did not separate.

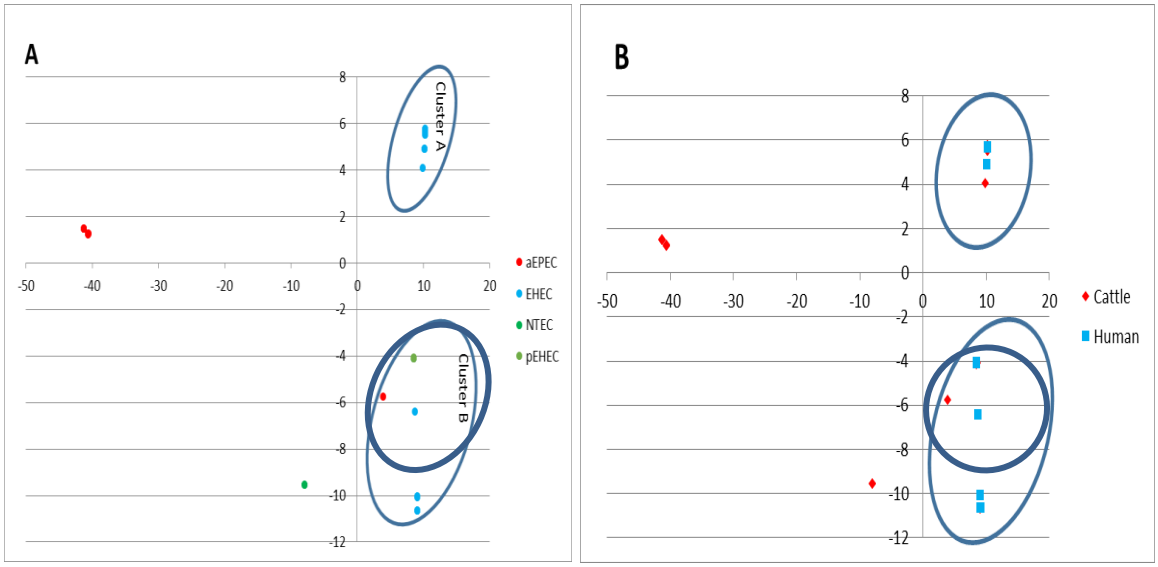


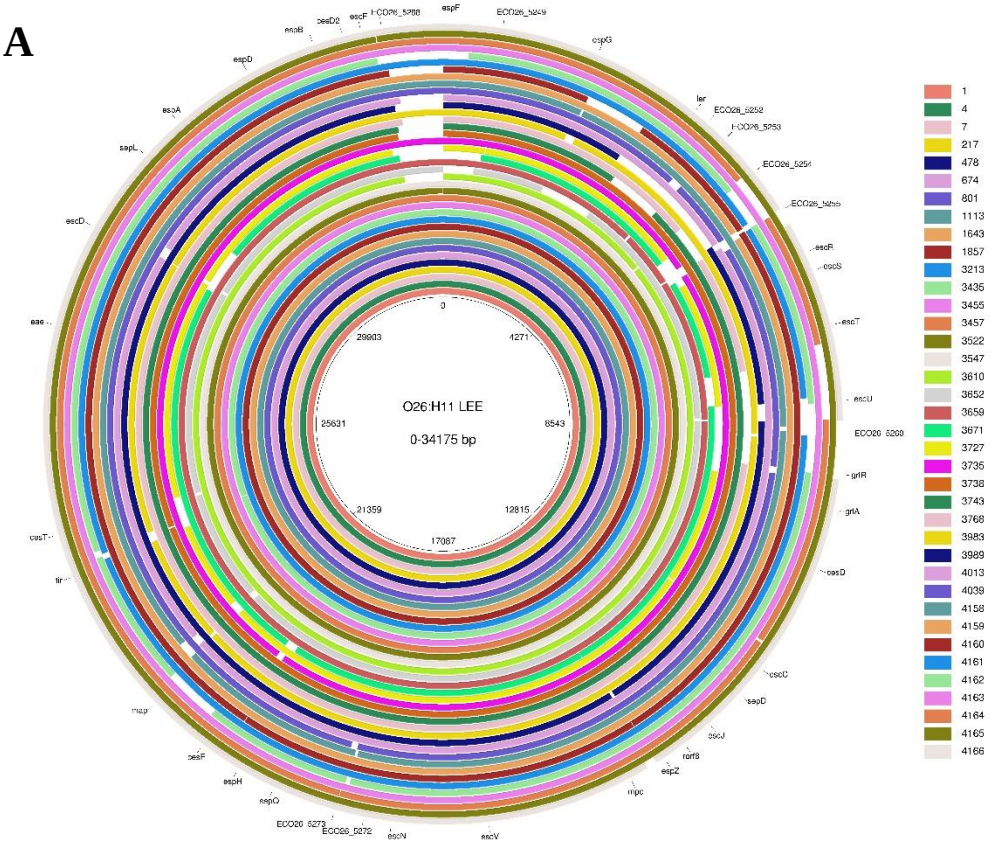
Figure 5-6 A & B. PCA analysis of SNPs using O26 reference strain (AP010953); A) labelled by pathotype; B) labelled by source. The single aEPEC strain and the three pEHEC O26 strains exhibiting EHEC characteristics are shown within shape A and B and are enclosed within circular shape boundaries.

5.4.6 SNPs within LEE

All strains were included in analysis except NTEC strains (EC3536 and 3946) as they lack the LEE PAI. Comparisons of LEE of O26 strains to LEE of reference strain (AP010953) revealed that most of *E. coli* O26 strains used in this study had very little variation in the LEE PAI compared to the O26 LEE reference (Figure 5.7A). Using Parsnp to identify SNPs, within LEE PI; 45 SNPs were identified within the LEE PI in *E. coli* O26 strains as shown in the dendrogram (Figure 5.7B). Investigation of SNP positions in the LEE PAIs segregated *E. coli* strains into pathotypes with some exceptions. For instance, mutations in the LEE at position 18135 (ECO26_5272) and at position 19328 (within *sepQ*) were identified in all aEPEC but absent in other pathotypes. Similarly a mutation located at position 30869 (*espD*) was observed only in pEHEC strains. *sepZ* had three mutational hotspots: seven aEPEC had a mutation at position 13702, 15 EHEC strains had the mutation at 13761 and in pEHEC strains

the mutation was found at position 13809. Interestingly, a group of EHEC (n=6) exhibited a number of unique SNPs that were not observed in other pathotypes or within other EHEC strains. These mutations were at position 13149 (*escI*), 23261(*tir* receptor) and 22478 (*tir* receptor). While clustering of strains based on pathotypes was evident, there was no evidence of clustering based on strain source.

A



B

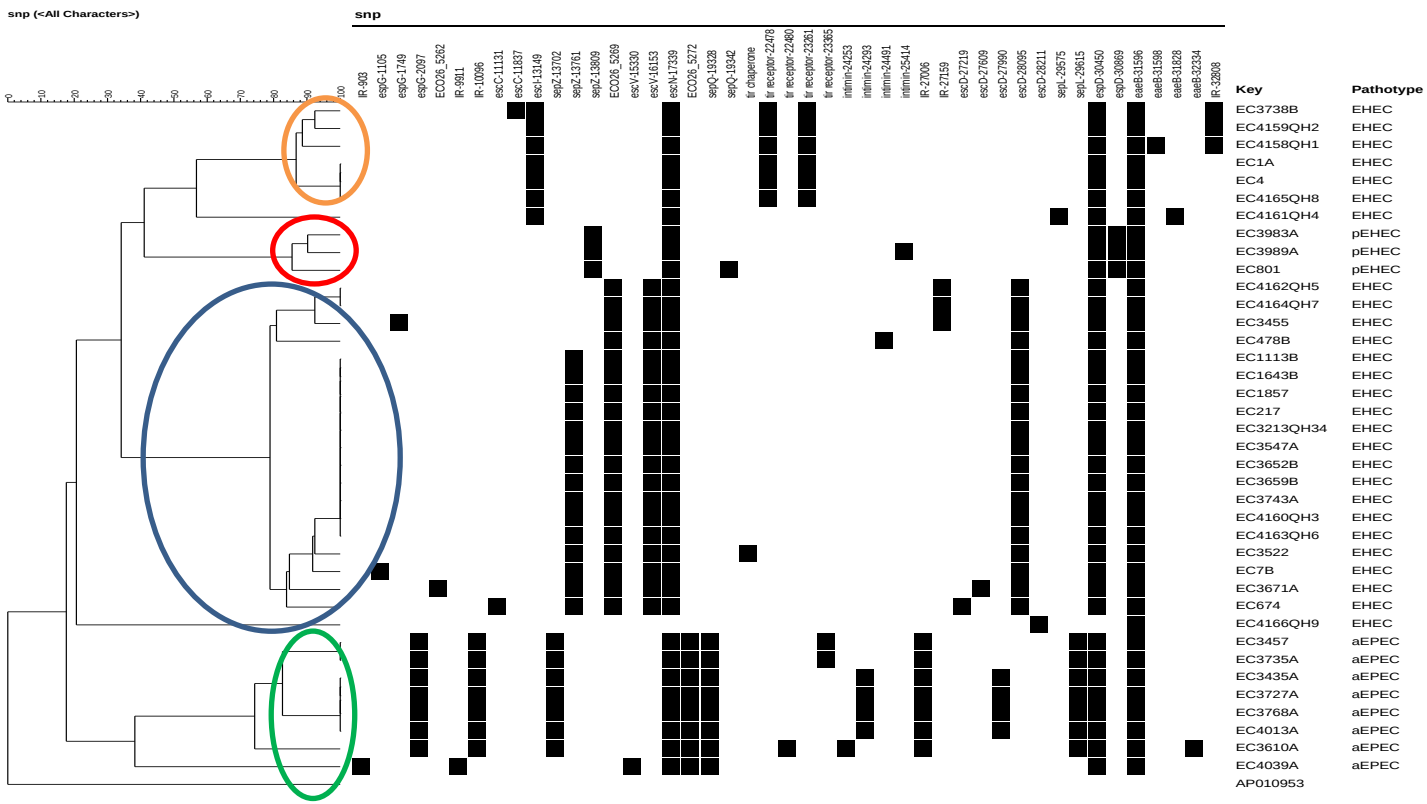


Figure 5-7. A) Comparisons of LEE of O26 strains to LEE of reference strain (AP010953); B) Dendrogram of SNPs occurred within LEE Pathogenicity Island of O26 strains. (B). EHEC strains are enclosed within blue oval boundary, pEHEC are within the red oval boundary and aEPEC are within the green oval boundary. NTEC strains were excluded from the analysis. EHEC (n=6) exhibited a number of unique SNPs that were not observed in other pathotypes or within other EHEC strains are within the orange oval boundary.

5.5 **Discussion**

In this study *E. coli* O26 strains from c and cattle sources in Australia were subjected to WGS data analysis utilizing a broad range of *in silico* typing methods. *In silico* serotyping successfully identified the H type for all strains but failed to assign 35% of strains into O type. Previously, these strains were shown to be O26 using conventional PCR targeted at *wzx* O26 (Chapter 2). BLAST searches of this gene against the *de novo* assemblies was conducted and identified partial sequence of *wzx* O26 which explains why the O type was not identifiable by SeroTypeFinder. Identification of O type is an essential part of the current STEC typing scheme when it's combined with other important EHEC attributes such as *stx* and *eae*. In addition, identification of SNPs within the O antigen gene cluster (*rmlA 30, wzx* 953 and *fnl1 88*) (Norman et al., 2012) proved to be useful for differentiation between EHEC/EHEC related strains from non-EHEC (Chapter 2) (Lajhar et al., 2017, Norman et al., 2012). However, if only a partial sequence of the gene of interest was found; an observation which was true for *wzx*, such an analysis would not be possible using short-read sequencing technology. It is expected that further developments in NGS technology will reduce the occurrence of these issues through the generation of substantially longer-read lengths.

The combination of WGS and virulence profiling of *E. coli* O26 strains permits greater understanding of the genetic composition of strains and enables relationships between strains to be assessed. The phylogenetic analysis using *chuA*, *TspE4.C2* and *yjaA* (Clermont et al., 2000) was conducted; and in contrast to other studies (Franz et al., 2015, Girardeau et al., 2005) which showed that EHEC belonging mainly to group A were *eae* negative (Girardeau et al., 2005) or were *stx2+*, and *ehx+*, all pathotypes in the current study were assigned into group A regardless

of their virulence profiles. Furthermore, in agreement with the other studies (Bugarel et al., 2011a, Miko et al., 2010) strains that exhibited the virulence gene profiles of EHEC but lacked *stx* (i.e. pEHEC, a single aEPEC; EC4039A) shared the same *arcA2* type with EHEC. Additionally, the finding that *espK*, *ureC*, *ureD*, *z2098* and *z2099* were present only in EHEC, pEHEC and a single aEPEC (EC4039A) is in accordance with previous studies (Delannoy et al., 2013b, Delannoy et al., 2013a, Delannoy et al., 2016) which showed that these virulence markers could discriminate EHEC as well as EHEC-like strains from other pathotypes.

Overall, strains of the same pathotype did not separate out based on source thereby confirming that cattle are a reservoir of EHEC O26 that can cause clinical illness. Nonetheless there were two contrasting observations between EHEC of human and cattle strains that are noteworthy. The Tir cytoskeleton coupling protein (*tccP*) is linked to severity of disease (Chase-Topping et al., 2012, de Boer et al., 2015) and in this study 40% of EHEC clinical strains possessed the gene which was not found in EHEC cattle strains. It is possible that the clinical disease experienced by these patients may have been more severe although information relating to HUS and HC occurrence is not known. Furthermore, the EHEC factor for adherence (*efa1*) was overrepresented in EHEC from cattle (58.82%) compared to that from clinical cases (10%) and is in contrast to previous studies (de Boer et al., 2015, Ferdous et al., 2016) that found higher prevalence of *efa1* amongst strains causing severe outcomes or bloody diarrhoea than those resulting in less severe outcomes. Conflicting results might be due to differences in disease outcomes, the low number of representative strains of *E. coli* O26 investigated, and absence of cattle strains in those studies.

The prevalence of AMR genes in Australian strains was also assessed and resistance to antimicrobials of critical important and highest priority to human health including cephalosporins (3^{rd}, 4^{th} and 5^{th} generation), glycopeptides, macrolides, ketolides, polymyxins and quinolones was not observed; reflecting the restriction on the use of antimicrobials of high importance to human medicine in food-producing animals and their infrequent use for treatment of gastroenteritis in clinical cases. Consistency between observed and predicted AMR was also observed demonstrating the usefulness of AMR prediction based on WGS (Holmes et al., 2015, Gonzalez-Escalona and Toro, 2016). Despite that, there were instances in this study where the genotypic and phenotypic results did not match. While AMR genotypes were identified by ResFinder based WGS analysis, the genes may not be expressed and therefore would not be detected by an AMR phenotypic assay.

Furthermore, the prevalence and the distribution of plasmids among *E. coli* O26 strains and within pathotypes using the PlasmidFinder tool provided further insight into the *E. coli* O26 population and relationship between strains. Clustering of *E. coli* O26 pathotypes obtained with plasmid typing was in agreement with other typing methods used in this study thereby supporting the occurrence of micro-evolutionary events within *E. coli* O26 strains in which horizontal gene transfer of mobile genetic elements such as plasmids contributes to the evolution of *E. coli* pathotypes. It's noteworthy that although the aEPEC strain namely EC4039A clustered with EHEC, it did not carry IncB/O/K/Z replicon (identified pO26-CRL plasmid) which would explain why this strain was negative for genes (*ehx*, *espP*, *katP*) carried on pO26-CRL plasmid and that were present in EHEC and pEHEC strains.

As previously mentioned, WGS was utilised to gain insights into *E. coli* O26 population structure and relationship between strains via conducting MLST, MLVA and pangenome analysis. Irrespective of the method used, results presented in this study revealed general clustering of EHEC-like (pEHEC and EC4039A) with EHEC with no further segregation based on source (human clinical or cattle). This was further highlighted by pEHEC and EHEC strains all being classified as ST21 by MLST or clustering of pEHEC strains and the aEPEC strain EC4039A with EHEC by MLVA. The finding that pEHEC and EHEC belong to the same MLST was demonstrated before in a USA study (Gonzalez-Escalona and Toro, 2016). In addition, the observation obtained with MLVA was in agreement with Brandal et al. (2012) who found that MLVA was able to distinguish EHEC/pEHEC from aEPEC but could not discern EHEC from genetically closely related strains. The relationship between *E. coli* O26 strains was further elucidated using LEE- and WG-SNPs assessments. While both SNP analysis methods identified subpopulations within EHEC that had unique SNPs from other strains of EHEC, WG-SNP analysis identified a subpopulation within EHEC that were closely related to pEHEC and the aEPEC strain EC4039A confirming that they shared the same phylogenetic cluster with EHEC. Although the rate at which these strains acquire *stx* in the environment and become EHEC is not known, they are likely to be precursors to strains that ultimately cause human disease and therefore further investigation is required. Together, these results provide evidence for the utility of WGS based SNP typing which has been utilised widely for *E. coli* O157 and to less extent with O26 and proved useful for typing other pathogens (Gonzalez-Escalona and Toro, 2016, Holmes et al., 2015, Michelacci et al., 2016).

5.6 **Conclusion**

In conclusion, this study utilised WGS based typing methods to gain insights into the population structure of *E. coli* O26 and the evolutionary relationships between strains derived from clinical cases and cattle from Australia. Data presented here identified that *E. coli* O26 prevalent in Australian cattle belong to ST21 (pEHEC and EHEC), ST 29 (aEPEC) or ST10 (NTEC). Greater understanding of the genetic composition of strains enables relationships between them to be assessed. A combination of molecular markers including *espK*, *ureC*, *ureD*, *z2098* and *z2099* could discriminate EHEC /EHEC-like strains (pEHEC and EC4039A) from other pathotypes. Overall, general agreement between different typing schemes was obtained with EHEC and EHEC-like routinely assigned to the same cluster. Consistent with epidemiological evidence in Australia that contact with animals on farm, work with raw meat and consumption of corned beef (McPherson et al., 2009), the schemes did not identify distinct features that could differentiate strains based on source suggesting that cattle are an important reservoir of clinical EHEC O26:H11 strains in Australia, although other risk factors for human infection with O26 have been reported in Australia (McPherson et al., 2009). Overall, while there appears to be overlap between clinical and cattle strains, all of the EHEC O26 included in this study are *stx1* and are therefore likely to be less virulent/ pathogenic than the recently described *stx2* clones that have appeared across Europe (ST29*/ stx2a/ ehxA+/ katP- / espP- /etpD+) or (ST29/ stx2d/ ehxA-/ katP-/ espP-/ etpD+).*

Chapter 6

General Discussion and Future Directions

6.1 **Summary of Thesis Objectives**

The purpose of this thesis was to phenotypically and genotypically characterise *E. coli* O26 strains from human and cattle sources in Australia. The specific aims of this study were to:

1. Characterise a collection of temporally and spatially separated Australian *E. coli* O26 isolates from human and cattle sources to identify a subset of strains that can be used for subsequent assays (Chapter 2).

2. Determine the response of the subset of *E. coli* O26 strains in planktonic form of growth to a range of antimicrobials and sanitisers (Chapter 2).

3. Determine the capacity of O26 and other pathotypes to form biofilms and explore the influence of pathotype, cells surface hydrophobicity, and phenotypic and genotypic attributes on biofilm formation (Chapter 3).

4. Determine the influence of biofilms on a strain's sensitivity toward sanitisers (Chapter 3).

5. Characterise Shiga-toxin bacteriophage insertion sites for EHEC strains and explore the ability of *stx*-negative strains to acquire the bacteriophage and establish a lysogen (Chapter 4).

6. Sequence and subtype *E. coli* O26 strains and conduct comparative genome fingerprinting to compare and contrast between human and cattle sourced strains and between pathotypes (Chapter 5).

6.2 **Major Findings and Contribution of This Thesis**

E. coli O26 is the second most frequently detected EHEC worldwide capable of causing disease in humans ranging from mild diarrhoea to more severe outcomes such as HUS. Isolation of big six EHEC including O26, O45, O103, O111, O121, O145 from beef cattle and beef cattle products and identifying them as a source of EHEC O26 in cases of foodborne disease has resulted in changes to regulations relating to the supply of beef products into export markets (USDA and FSIS, 2012). The detection of *E. coli* O26 strains in various environments including food, processing equipment and food contact surfaces and identifying them as a causative agent for a number of foodborne outbreaks creates the need for implementing prevention strategies to control this pathogen. Food producers and processors are able to identify critical control points during production and processing and can use a range of antimicrobial agents to assist in controlling this organism or to provide benefit to the food production system. In comparison to *E. coli* O157, relatively little is known about the ability of *E. coli* O26 to persist in processing environments or to develop resistance to antimicrobials. Similarly, it is not completely understood which suite of bacterial virulence determinants are most necessary for *E. coli* O26 to cause disease in humans although strains recovered from clinical samples typically possess *stx1*, *stx2* and *eae* which is necessary for bacterial colonisation of the gut. Overall, this study was performed to gain insight into the characteristics of *E. coli* O26 circulating in Australian cattle and that associated with human disease and to explore whether a range of possible interventions (acids and disinfectants) applied in meat and food industry processing environments are effective against this pathogen under certain conditions. Comparisons between source and/or pathotype

were conducted to assist in identifying factors that could contribute to the ability of O26 strains to survive stress intervention encountered in food industry and subsequently cause disease.

Initial characterisation efforts identified that *E. coli* O26 can belong to a range of pathotypes including: EHEC, aEPEC, pEHEC and NTEC on the basis of virulence markers (*stx*, *eae*, *ehx*, *ecf, rmlA* SNP and *bfpA*) and two main clusters by PFGE. AMR results were consistent with previously published Australian reports with low levels of resistance to antimicrobials of critical importance for human health identified thereby reflecting the effectiveness of regulatory measures in controlling the development of AMR in food producing animals in Australia (Barlow and Gobius, 2008, Barlow et al., 2015). When the initial characterisation results were combined with spatial and temporal data a subset of 40 out of 88 isolates comprising ten human and 30 cattle *E. coli* O26 strains were selected for subsequent analysis.

EHEC O26 contaminating cattle meat and other beef or dairy products can cause foodborne illness by evading or resisting the interventions designed to control their growth or ability to persist within the food industry processing environment or on the meat surface. The response of *E. coli* O26 strains in planktonic (Chapter 2) or biofilm forms (Chapter 3) to a range of antimicrobials and sanitisers and the possible influence of pathotype or strain source was assessed. The overall results of the study identified that survival of pathogens to disinfectants varies depending on whether the cells are in a planktonic or biofilm state and which stressor is applied. Chapter 2 demonstrated that in the planktonic state, both QAC compounds (0.45% Kwiksan, 1% Profoam) and the peracetic acid based sanitiser (1% Topactive Des. which are commonly used in the food industry and clinical settings inhibited the growth of *E. coli* O26 of all pathotypes at proposed industry working concentrations validated for O157. Whereas all

pathotypes survived treatment with the recommended concentrations of hypochlorite based sanitisers (Dairy Chlor12.5% and 1.6% Maxifoam) and acid anionic sanitiser (0.4% Envirosan). It was also demonstrated that O26 strains of all pathotypes examined here had elevated MICs (1,024 to 4,096 µg/ml) to acetic, propionic, lactic and citric acids which are comparable to that of O157 strains assessed in previous studies (Beier et al., 2013, Kalchayanand et al., 2012) suggesting that interventions implemented against O157 may indeed be effective against other *E. coli* pathotypes or even serogroups. It also implies that similar to O157, *E. coli* O26 strains of different pathotypes utilise a number of mechanisms to prevent the lethal effect of sanitisers and acidic stresses. Finally, *E. coli* O26 in planktonic state did not demonstrate variability in resistance when source or pathotype was considered.

The capacity to form biofilms has been described as a key mechanism by which susceptible strains resist the effects of sanitisers (Vogeleer et al., 2015, Wang et al., 2012). Assessing the capacity of strains of different pathotypes to form biofilm under static conditions on surfaces commonly used for food preparation and in food industry (Chapter 3) revealed that biofilm formation on one surface may not predict exhibition of this phenotype on another substratum and that incubation time has an impact on biofilm formation, in agreement with previous studies (Wang et al., 2012, Rivas et al., 2007). Low biofilm formation was observed for all strains after 24 hr whereas EHEC strains were more likely to form biofilms after 48 hr on polystyrene plates than aEPEC strains. However, the biofilm phenotype of all pathotypes was greatly enhanced after 72 hours regardless of the biofilm mass formed after 24 or 48h. It was also revealed that biofilm formation of different pathotypes was enhanced when stainless steel and glass slides were used as a contact surface for biofilm formation. However, different observations were

obtained when assessing the capacity for pellicle formation at the air-liquid interface where an opaque pellicle layer attached to the wall of a glass tube provides bacterial cells with oxygen from the air and nutrients from the liquid medium. Results indicated that pellicle formation was constrained primarily to EHEC isolates while it was absent from aEPEC suggesting that EHEC may have greater capacity to survive in a range of environments.

Several additional factors that could enhance or limit biofilm formation were evaluated in this study (Chapter 3). It was observed that a prophage insertion in *mlrA* (*yehV*) and failure in producing ECM (curli and cellulose) appears to prevent or lower biofilm formation in the majority of EHEC whereas in aEPEC the lack of ECM components (SAW morphotype) and lack of motility were the main factors more likely to be associated with reduced biofilm formation. Chapter 3 also showed that biofilm formation occurs in other *E. coli* O26 pathotypes including pEHEC and NTEC with NTEC strains displaying BDAR (curli) and strong biofilm formation on all surfaces at 25^{o}C. Moreover, a novel finding of this study was the first description of the biofilm attributes of pEHEC isolates which were found to express BDAR (curli) or PDAR (cellulose) and form more biofilm on stainless steel surfaces than other surfaces. The possible influence of the cell surface hydrophobicity on biofilm formation was also investigated; however, no correlation was found. While there are caveats to the results observed in this study, the utility of this study is to provide initial insights into factors that could possibly influence biofilm formation by *E. coli* O26 and then the effect of this phenotype on tolerance to disinfectants. Further studies that represent the food and meat plant situation and consider the effect of co-existence with other microorganisms, presence of organic residues on food surfaces

and resistance or adaptation to disinfection are required (Reviewed in (Van Houdt and Michiels, 2010))

The availability of genome sequence data (Chapter 5) allowed further investigation of the role of autotransporters, fimbriae encoding genes and the occurrence of RpoS mutation in biofilm formation (Chapter 3-Supplementary information). It was found that mutations in *RpoS* was not common amongst the O26 isolates and was observed only in two clinical EHEC isolates that had previously been shown to have no capacity for pellicle formation at the air liquid interface and low biofilm forming capacity on glass slides. Furthermore, the current study did not identify any linkage between the presence of the autotransporter and fimbriae-encoding genes and the ability to form biofilms as there were no differences in the carriage of these genes between isolates or pathotypes. Finally, the biofilm development *YmgB*/*AriR* family protein was found in all pathotypes except aEPEC which may explain the low biofilm forming capacity of aEPEC that also lacked motility and ECM. Differences between EHEC clinical and cattle strains in their capacity for biofilm formation or association with specific biofilm forming attributes were not observed which may suggest that cattle isolates represent a source of biofilm-forming bacteria that might contaminate processing environments and colonise meat surfaces and subsequently cause human infection.

Insights into the capacity of *E. coli* O26 isolates to from biofilms enabled further experiments to be conducted which examined the effect of the three sanitisers, previously shown to successfully control the growth of their planktonic counterparts (Chapter 2), on biofilms (Chapter 3). All sanitisers were unable to kill all cells of a biofilm regardless of the amount of biofilm formed, morphotype expressed (curli or cellulose), pathotype or the strain source, indicating that

encasement of bacterial cells in a biofilm matrix provides protection against commonly used sanitisers. Since the three sanitisers approved for use in the Australian food industry at their recommended concentration inhibited the growth of O26 in planktonic state, and the vast majority of strains did not form biofilm after 24 hr, regular and proper sanitisation might be effective in preventing the formation of biofilms in food production and processing environments, although a study that considers food industry environment is required to confirm this conclusion

The characterisation of strains that belong to a number of *E. coli* O26 pathotypes highlights many of the common and distinguishing features that exist (Chapter 2 and Chapter3). As previously mentioned in Chapter 1, one of the defining features separating *E. coli* pathotypes is the carriage of *stx*. The potential for *stx* to be gained by *E. coli* O26 and the impact on diagnosing foodborne disease and testing for STEC as part of the export red meat testing program is poorly understood for *E. coli* O26 (Chapter 4). Defining the SBI in EHEC and understanding the capacity for non EHEC pathotypes to acquire *stx* can assist in understanding the risk that these organisms pose to human (Mellor et al., 2015, Bielaszewska et al., 2007). The study demonstrated for the first time the SBI for EHEC O26 from Australian clinical and cattle sources and for their corresponding lysogens. Shiga toxin prophage was found to integrate into a novel insertion site near the *torS* gene but not in the previously described *torS-torT* intergenic region of *E. coli* O103 (Ogura et al., 2007). Other SBIs identified included *wrbA* in a single clinical EHEC strain and *yehV* in seven EHEC strains. The *wrbA* and *yehV* have been commonly associated with *stx2* and *stx1* respectively with *yehV* routinely occupied by prophages other than Stx (Ogura et al., 2007, Bielaszewska et al., 2007). It was also found that these loci were intact in *stx*-

negative strains investigated in this study, a factor which was thought to facilitate the occupancy of these insertion sites by Stx phage. However, when transduction experiments were conducted the generation of a stable lysogen was not possible despite this apparent susceptibility. Although this study was unable to detail the ability or frequency at which *stx* phage are acquired by *stx*-negative O26 strains, there remains a need to continue to be aware of the possibility of Stx acquisition and its effect on diagnostic systems. In addition to the identification of SBI's of *stx*-containing strains, all strains were analysed for the presence of a wide range of phage integrases previously associated with enteric bacteria. Phage integrase carriage (Chapter 4-Supplementary information) was shown to be pathotype specific and likely promotes the differing genetic profiles observed between pathotypes thereby reaffirming the role of bacteriophage in the evolution of pathogenic *E. coli*.

Stx phage induction plays an important role in promoting *stx1* transcription, amplifying *stx1* copy number and/or allowing Stx release by phage-mediated lysis mechanism to consequently increase Stx toxin synthesis and release (Wagner et al., 2002). In order to further our understanding of the role bacteriophages play in generating diversity within bacterial populations, broader analysis was conducted on a subset of strains that were able to generate stable lysogen in K-12 strains (Chapter 4). Comparative analysis of Stx phage induction (using spectrophotometer), *stx* copy number (using QuantStudio dPCR) and toxin production (using ELISA) identified variability within O26 EHEC. Prophage induction and *stx* copy number levels (Using QuantStudio dPCR), were higher in MMC compared to NMMC induced cutlers. In addition, using ELISA, lysogens were found to produce greater quantities of Stx than their parental counterparts with no correlation identified between the Stx production levels in the

EHEC parent strains and lysogens. Comparable Stx phage induction, *stx* copy number and Stx production levels were observed between cattle and cattle strains confirming that cattle represent an important reservoir of strains with the potential to cause human foodborne illness.

During the course of this study substantial advances were made in the cost and ease of application of next generation sequencing (NGS) technique that permitted the whole genome sequencing (WGS) of the subset of 40 O26 strains (Chapter 5). WGS data presented in the thesis revealed that *E. coli* O26 pathotypes belong to H11 except NTEC strains which express H32. *In silico* analysis of genetic markers previously shown to be associated with EHEC demonstrated that the majority of Australian EHEC O26 belong ST21, harbour *stx1a, eae, espK, Z2099, ureD, ureC, Z2098, ehx, katP* and *espP*. The relationship between clinical and cattle strains was further confirmed and general agreement between different *in silico* typing schemes (MLVA, pangenome analysis and LEE- and WG-SNP analysis) was obtained; suggesting that cattle are a major reservoir of *E. coli* O26 strains associated with clinical disease. Whilst strains belonging to the EHEC group are of most interest because of their link to human clinical illness, pEHEC of O26 although lacking *stx*, appear to contain the necessary attributes of EHEC strains. This group exhibited EHEC virulence determinants, plasmid profiles and clustered with EHEC by MLST (ST21), MLVA, pangenome analysis and SNPs. In addition, in agreement with previous studies (Delannoy et al., 2013b, Delannoy et al., 2013a, Delannoy et al., 2016), the combination of genetic markers including *espK*, *ureC*, *ureD*, *z2098* and *z2099* facilitates the identification of EHEC-like strains that appear to differ from EHEC by the lack of *stx*. This study also demonstrated that the majority aEPEC are divergent from other pathotypes based on sequence type (ST29) and virulence as well as plasmid profile. Finally, NTEC had distinct MLST (ST10),

MLVA, PCA of pangenome and SNP, exhibited absence of EHEC virulence determinants and all EHEC plasmid profiles.

6.3 **Conclusion**

This is the first study of its type to comprehensively describe important phenotypic and genotypic characteristics of *E. coli* O26 and gain insights into the relationship between strains. Analysis revealed that O26 belong to a range of pathotypes including EHEC, pEHEC, aEPEC and NTEC. Factors affecting survival of *E. coli* O26 to sanitisers were evaluated and it was determined that survival varies depending on whether the cells are in a planktonic or biofilm state and which stressor is applied. The role that Stx1-phage plays in bacterial evolution and diversity was highlighted within the study with a novel SBI for EHEC O26 identified. Although this study was unable to detail the ability or frequency at which *stx1*-phage are acquired by *stx*-negative O26 strains, there remains a need to continue to be aware of the possibility of Stx acquisition and its effect on diagnostic systems. Finally, the use of WGS provides evidence of the continuous evolution and plasticity of the genome of this organism and its ability to undergo genetic rearrangements and further confirms that cattle are a reservoir of EHEC strains capable of causing human disease. Whilst maintaining a focus on EHEC is essential, the contribution of other *E. coli* pathotypes in their reservoir and surrounding environment requires ongoing monitoring. Continued evaluation of bactericidal substances for their effectiveness to control this economically important pathogen to the food industry is required.

6.4 Future directions

6.4.1 Analysis of transcriptome response to disinfectants.

The effect of sanitisers on *E. coli* O26 in planktonic state was studied and it was demonstrated that certain sanitisers were more effective than others. Analysis of gene expression patterns after exposure to disinfectants for 2 min and 10 min and comparisons of the response to effective and non-effective concentrations of the same disinfectant can be performed to analyse the genome-wide transcription changes. Insight into the molecular mechanism of response might assist in identifying unique mechanisms that O26 utilise to survive disinfectants which then can be targeted to reduce O26 survival in food industries.

6.4.2 Dissemination of *stx* genes by transduction within biofilms

The transduction of Stx1 bacteriophage to *stx*-negative O26 strains to generate a stable lysogen was attempted during this study but was unsuccessful. This process remains a key evolutionary step in the development of EHEC and further investigation is required. Previous studies (Solheim et al., 2013) have reported that acquisition of *stx* can occur within biofilms at both 20°C and 37°C and may provide opportunity to further assess the capacity of pEHEC to acquire Stx and become EHEC. Subsequent measuring of phage induction, infectivity level, host range, toxin production and expression can occur if the generation of lysogens is achieved.

6.4.3 Biofilm under conditions relevant to the food production chain

The assessment of biofilm forming capacity in the current study was completed at 25^{o}C on three surfaces: polystyrene, glass slide, stainless steel to facilitate comparison with other studies. However, it is critical to conduct experiments using conditions present in food processing environments and therefore the testing of alternative surfaces (e.g. Teflon and rubber) and at lower temperatures is required.

6.4.4 Biofilm formation and stress resistance in mixed community biofilm

Biofilms can be made up of a highly diverse community which compete for space and nutrients. In the current study, while the biofilm characteristics of strains of each pathotype was determined and resistance to disinfectant intervention was observed, understanding of biofilm forming capacity of O26 strains could be further elaborated by studying the effect of mixed species community biofilm on development of biofilm, overgrowth of one pathotype over the other, morphotype structure and stress response.

6.4.5 Stx phage sequencing

Determination of phage structure and insertion site is an ongoing issue that plagues STEC characterisation efforts. There are now a number of sequencing systems that can achieve long sequencing reads with uniform coverage that might make it possible to identify the exact insertion site and sequence of stx bacteriophage. In addition, it will be interesting to identify

factors that account for the broad host range leading to the emergence of new *stx*-positive pathogens. Furthermore, the sequencing of Stx phage may help to determine if the carriage of either an extra-antirepressor gene or an anti-repression operon by the Stx1 phage had a role in the different toxin production level observed as has been previously demonstrated with O157 (Sim et al., 2015).

6.4.6 Effectiveness of antibiofilm agents against *E. coli* O26 biofilms.

As all sanitisers used in the study were not able to completely kill cells within biofilms, additional antibiofilm agents might be promising in control the occurrence of biofilms in food processing establishments. Antibiofilm agents that could be used include proteinase K, cellulase and sonorensin. Previous studies (Chopra et al., 2015, Vogeleer et al., 2015) have demonstrated that some biofilm forming *E. coli* strains were sensitive to treatment with proteinase K and that sonorensin can significantly control the growth of *Staphylococcus aureus* on chicken meat and tomato samples as well as inhibit their attachment and biofilm formation on polystyrene plates.

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Appendix 1 Virulence genes detected by CGE web-based tools.

Group	Gene Name	Description
Family-,genus- and species-specific genes	*gad*	Glutamate decarboxylase
	ipaH9.8	Invasion plasmid antigen
Genes encoding virulence factors -Shiga toxins	*stx1A*	Shiga-like toxin 1 A-subunit
	stx1B	Shiga-like toxin 1 B-subunit
	stx2A	Shiga toxin 2 subunit A
	stx2B	Shiga toxin 2 subunit B
	stx1a	Shiga toxin 1 variant a (Scheutz et al. 2012)
	stx1c	Shiga toxin 1 variant c
	stx1d	Shiga toxin 1 variant d
	stx2a	Shiga toxin 2 variant a (Scheutz et al. 2012)
	stx2b	Shiga toxin 2 variant b
	stx2c	Shiga toxin 2 variant c
	stx2d	Shiga toxin 2 variant d
	stx2e	Shiga toxin 2 variant e
	stx2f	Shiga toxin 2 variant f
	stx2g	Shiga toxin 2 variant f
Genes encoding virulence factors - toxins	*astA*	Heat-stable enterotoxin 1
	cba	Colicin B
	ccI	Cloacin
	cdtB	Cytolethal distending toxin B
	celb	Endonuclease colicin E2
	cma	Colicin M
	cnf1	Cytotoxic necrotizing factor
	hlyE	Avian *E. coli* hemolysin
	ipaD	Invasion protein Shigella flexneri
	ltcA	Heat-labile enterotoxin A subunit
	mchB	Microcin H47 part of colicin H
	mchC	MchC protein
	mchF	ABC transporter protein MchF
	mcmA	Microcin M part of colicin H
	pet	Autotransporter enterotoxin
	sat	Serine protease autotransporters of Enterobacteriaceae
	senB	Plasmid-encoded enterotoxin
	sta1	Heat-stabile enterotoxin ST-Ia
	stb	Heat-stabile enterotoxin II

	subA	Subtilase toxin subunit
	toxB	Toxin B
	virF	VirF transcriptional activator
Genes encoding virulence factors - adhesins	*eae*	Intimin
	eae epsilon	Intimin subtype epsilon (AJ303141)
	eae gamma	Intimin subtype gamma (AF071034)
	eae theta	Intimin subtype theta (FM872418)
	eae beta	Intimin subtype beta (ECU59503)
	efa1	EHEC factor for adherence
	espB	Secreted protein B
	fasA	Fimbrial 987P/F6 subunit
	fedA	Fimbrial protein F107 subunit A
	fedF	Fimbrial adhesin AC precursor
	fim41a	Mature Fim41a/F41 protein
	iha	Adherence protein
	nfaE	Non-fimbrial adhesin
	saa	STEC autoagglutinating adhesin
Genes encoding virulence factors - fimbrae	*bfpA*	Major subunit of bundle-forming pili
	cfa_c	Colonization factor antigen I
	cofA	Longus type IV pilus subunit
	f17A	Subunit A of F17 fimbrial protein
	f17G	Adhesin subunit of F17 fimbriae
	fanA	Involved in biogenesis of K99/F5 fimbriae
	K88ab	K88/F4 protein subunit
	lngA	Longus type IV pilus
	lpfA	Long polar fimbriae
	perA	EPEC adherence factor
	prfB	P-related fimbrial regulatory gene
	sfaS	S-fimbrial minor subunit
Genes encoding virulence factors - secretion systems	*cif*	Type III secreted effector
	espA	Type III secretion system
	espC	Serine protease autotransporters of Enterobacteriaceae
	espF	Type III secretion system
	espI	Serine protease autotransporters of Enterobacteriaceae
	espJ	Prophage-encoded type III secretion system effector
	etpD	Type II secretion protein
	nleA	Non-LEE-encoded effector A
	nleB	Non-LEE-encoded effector B
	nleC	Non-LEE-encoded effector C
	tccP	Tir cytoskeleton coupling protein
Genes encoding virulence factors -	*eatA*	Serine protease autotransporters of Enterobacteriaceae (SPATE)
	epeA	Serine protease autotransporters of Enterobacteriaceae

SPATE (serine protease autotransporters)	*espP*	Putative exoprotein precursor
	pic	Serine protease autotransporters of Enterobacteriaceae
	rpeA	Serine protease autotransporters of Enterobacteriaceae
	sepA	Serine protease autotransporters of Enterobacteriaceae
	sigA	Serine protease autotransporters of Enterobacteriaceae
	tsh	Serine protease autotransporters of Enterobacteriaceae" temperature - sensitive haemagglutinin"
	vat	Serine protease autotransporters of Enterobacteriaceae
Genes encoding virulence factors - miscellaneous	*ehxA*	Enterohaemolysin
	ireA	Siderophore receptor
	iroN	Enterobactin siderophore receptor protein
	iss	Increased serum survival
	katP	Plasmid-encoded catalase peroxidase
	tir	Translocated intimin receptor protein

Appendix 2 Publications Related to this Thesis.

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BMC Microbiology

RESEARCH ARTICLE Open Access

Survival capabilities of *Escherichia coli* O26 isolated from cattle and clinical sources in Australia to disinfectants, acids and antimicrobials

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Abstract

Background: After *E. coli* O157, *E. coli* O26 is the second most prevalent enterohaemorrhagic *E. coli* (EHEC) serotype identified in cases of foodborne illness in Australia and throughout the world. *E. coli* O26 associated foodborne outbreaks have drawn attention to the survival capabilities of this organism in a range of environments. The aim of the present study was to assess the ability of *E. coli* O26 to survive the effects of disinfectants, acids and antimicrobials and investigate the possible influence of virulence genes in survival and persistence of *E. coli* O26 from human and cattle sources from Australia.

Results: Initial characterization indicated that *E. coli* O26 are a genetically diverse group that were shown to belong to a number of pathotypes. Overall, 86.4% of isolates were susceptible to all antimicrobials tested with no significant differences in resistance observed between pathotypes. A representative subset of isolates ($n = 40$) were selected to determine their ability to survive disinfectants at proposed industry working concentrations and acid stress. Profoam, Kwiksan 22, and Topactive DES. were able to inhibit the growth of 100% of isolates. The remaining three disinfectants (Dairy Chlor 12.5%, Envirosan and Maxifoam) were not effective against the subset of 40 *E. coli* O26. Finally, elevated MICs (1,024 to 4,096 μg/ml) of acetic, propionic, lactic, and citric acids were determined for the majority of the isolates (85%).

Conclusions: Australian *E. coli* O26 isolates belong to a range of pathotypes that harbor differing virulence markers. Despite this, their response to antimicrobials, disinfectants and acids is similar confirming that stress response appears unrelated to the presence of EHEC virulence markers. Notwithstanding, the tolerance to disinfectants and the elevated acid MICs for EHEC and the other *E. coli* O26 pathotypes examined in this study may contribute to bacterial colonization on food contact surfaces and subsequent foodborne illness caused by this pathogen.

Keywords: *E. coli* O26, Virulence marker, Pathotype, Antimicrobial agent, Disinfectant, Organic acid

Background

Enterohaemorrhagic *Escherichia coli* (EHEC) strains have been associated with a number of food-borne outbreaks which have led to life threatening sequelae such as hemolytic uremic-syndrome (HUS) and hemolytic colitis (HC) [1–3]. Epidemiological surveillance indicates that *E. coli* of O157:H7 serotype is the most frequently encountered EHEC implicated in sporadic and outbreak cases of illness [4]. However, other non-O157 serotypes such as O26:H11, O45:H2, O103:H2, O111:H8, O121:H19, O145:H28 and their non-motile forms have emerged and are now considered an important cause of human infection resulting in HUS [5]. Among the non-O157 serotypes, *E. coli* O26:H11 is one of the major serotypes of concern [3, 6].

Although it is not completely understood which suite of bacterial virulence determinants are most necessary for *E. coli* O26 to cause disease in humans, isolates recovered from human clinical samples typically possess

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