Detection of Shiga-toxigenic and enteropathogenic *Escherichia coli* in diarrhoeic stools

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A thesis submitted for the degree of Master of Medical Laboratory Science

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Abstract

Shiga-toxigenic *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC) are recognised to be significant contributors to intestinal and extra-intestinal disease and infantile diarrhoea worldwide. In New Zealand, STEC serotype O157:H7 is a notifiable pathogen. The routine use of sorbitol MacConkey (SMAC)-based agar to detect O157:H7 fails to detect many potentially pathogenic non-O157 *E. coli* serogroups and EPEC is not currently under surveillance. As a result, the contribution of these pathogens to diarrhoeal disease is likely to be underrepresented in governmental surveillance reports. The intent of this project was to develop a multiplex qRT-PCR TaqMan assay suitable for the detection of STEC (stx1 and stx2) and EPEC (*eaeA*/intimin) marker genes, and to use this assay to survey diarrhoeic stool samples sourced from Dunedin Hospital. Prevalences of 1.53% and 4.41% (95% CI) were found for STEC and EPEC respectively, with 75% of stx+ samples possessing STEC belonging to serogroups other than O157. A trend towards an increased rate of STEC infection amongst samples also tested for *Helicobacter pylori* was also noted, indicating possible confusion of symptoms by clinicians. The project also tested a number of DNA extraction methods and pre-enrichments to further adapt the assay to high-throughput diagnostic practice. The inexpensive Chelex-100 resin performed satisfactorily when compared with phenol-chloroform and PrepMan Ultra methods. Trypticase soy broth was found to offer the most consistent pre-enrichment for STEC compared to other commonly used non-selective liquid culture media. A 3 hour pre-enrichment of spiked faecal samples was found to boost the sensitivity of the qRT-PCR method to detect $2 \times 10^2$ CFU per g of faeces.
Acknowledgments

I wish to extend my sincerest gratitude to my supervisors Dr. Heather Brooks, who has been a constant guide, mentor and sounding board in times of need, and my co-supervisor Dr. Rory O’Brien for his sage technical advice and for putting up with my inane questions. The food bribes probably helped. Without their input, this project would not have been possible and I owe my success to their assistance as much as my own effort.

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<td>A/E</td>
<td>Attaching/effacing (lesions)</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BD</td>
<td>Becton-Dickinson (company)</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain hear infusion broth</td>
</tr>
<tr>
<td>bp</td>
<td>Base-pairs</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention (US Federal Agency)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming units</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CT-SMAC</td>
<td>Sorbitol-MacConkey Agar, supplemented with cefixime and tellurite</td>
</tr>
<tr>
<td>DAEC</td>
<td>Diffusely-adhererent <em>Escherichia coli</em></td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose Nucleic Acid</td>
</tr>
<tr>
<td>EAEC</td>
<td>Enteraggregative <em>Escherichia coli</em></td>
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<tr>
<td>EC</td>
<td><em>E. coli</em> broth</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohaemorrhagic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EIEC</td>
<td>Enteroinvasive <em>Escherichia coli</em></td>
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<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
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<tr>
<td>ERL</td>
<td>Enteric Reference Laboratory (ESR)</td>
</tr>
<tr>
<td>ESR</td>
<td>Environmental Sciences and Research (a NZ Crown Research Institute)</td>
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<tr>
<td>fg</td>
<td>femtogram</td>
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<td>g</td>
<td>gram</td>
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<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>GC</td>
<td>guanine-cytosine content</td>
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<td>HC</td>
<td>Haemorrhagic colitis</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td><em>Helicobacter pylori</em></td>
</tr>
<tr>
<td>HUS</td>
<td>Haemolytic-uraemic syndrome</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable Bowel Syndrome</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>LAMP</td>
<td>Loop-mediated isothermal amplification</td>
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<tr>
<td>LEE</td>
<td>Locus of Enterocyte Effacement</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
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<td>---------</td>
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</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix assisted laser desorption ionization time-of-flight</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>Non-O157</td>
<td><em>Escherichia coli</em> (usually STEC) of serogroups other than O157</td>
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<tr>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PC2</td>
<td>Physical containment level 2</td>
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<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>pg</td>
<td>pictogram</td>
</tr>
<tr>
<td>PVPP</td>
<td>Polyvinylpolypyrrolidone</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>R²</td>
<td>Coefficient of determination</td>
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<tr>
<td>SCL</td>
<td>Southern Community Laboratories</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>Shiga-like toxigenic <em>Escherichia coli</em> (early name for STEC)</td>
</tr>
<tr>
<td>SMAC</td>
<td>Sorbitol-MacConkey Agar</td>
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<td>SOP</td>
<td>Standard operating procedure</td>
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<td>STEC</td>
<td>Shiga-toxigenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>TE</td>
<td>Tris-hydroxymethyl-aminomethane buffer fortified with EDTA</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris-hydroxymethyl-aminomethane buffer</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic Soy Agar</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic Soy Broth</td>
</tr>
<tr>
<td>TTP</td>
<td>Thrombotic thrombocytopenic purpura</td>
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<tr>
<td>µL</td>
<td>microlitre</td>
</tr>
<tr>
<td>µM</td>
<td>micromole</td>
</tr>
<tr>
<td>µm</td>
<td>micrometre</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
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1 Introduction

1.1 General Introduction

Shiga-toxigenic *Escherichia coli* (STEC) are a toxin-producing subset of the normally commensal enteric bacterium *E. coli* and are recognised to be a significant contributor to diarrhoeal disease worldwide. In the United States, STEC are considered among the most prolific foodborne pathogens (1), estimated to be responsible for at least 100,000 episodes of illness, 3,000 hospitalisations and 90 deaths annually (2, 3) at an estimated cost (as of 2009) of $478 million dollars (4). Much of the epidemiological and diagnostic efforts regarding STEC revolve around strains of the *E. coli* serotype O157:H7, known to be the serotype most commonly associated with progression to severe forms of gastrointestinal disease, such as haemorrhagic colitis (HC), and extra-intestinal disease, such as haemolytic uraemic syndrome (HUS) (5, 6).

In recent years, international researchers and surveillance networks have noted increases in the number of cases of diarrhoea and more severe symptoms caused by STEC belonging to serogroups other than O157 (collectively referred to as ‘non-O157’ STEC) (7). Non-O157 STEC are recognised to contribute to a significant proportion of STEC-related illness in first world countries (8), with some strains considered to have a pathogenic potential comparable to that of O157:H7 (8, 9). Much of the disease burden presented by non-O157 STEC remains under-reported in many countries (10, 11), mainly due to limitations in popular diagnostic techniques to detect these serogroups (2, 12).

*E. coli* O157:H7 related disease is notifiable in New Zealand, and national data accumulated since the first case of O157:H7 infection in 1993 indicates that the incidence of all reported STEC has been steadily increasing in ensuing years (13, 14). The isolation and subsequent reporting of *E. coli* O157:H7 by diagnostic laboratories is typically based upon plate culturing of faecal samples on Sorbitol MacConkey Agar (SMAC), which provides limited information on the presence of non-O157 STEC. The intent of this project was to design a practicable multiplex qPCR test procedure that could be used to identify both non-O157 and O157 STEC, and to use this to determine the prevalence of these bacteria within diarrhoeal stool samples sourced from Dunedin Hospital. A secondary objective was to investigate the incidence of the *E. coli* outer membrane...
protein intimin as a marker of the Locus of Enterocyte Effacement (LEE) pathogenicity island, which is a key virulence factor in infections of both STEC and enteropathogenic E. coli (EPEC).

1.2 Shiga-toxigenic Escherichia coli

STEC, also referred to as Vero-cytotoxigenic E. coli (VTEC) or Shiga-like toxigenic E. coli (SLTEC), are one of several gastrointestinal pathotypes of E. coli, a Gram-negative facultatively anaerobic bacterium that inhabits the mucous layer of the mammalian lower intestine (15, 16) A summary of all E. coli enteric pathotypes is provided in Figure 1. E. coli, as a species, are generally commensal bacteria that can cause opportunistic urinary tract infections, meningeal disease or enteric diseases depending on the virulence factors present (16). Several major E. coli pathotypes (such as STEC) have emerged in the evolutionary history of E. coli through the transfer of virulence factors from other bacterial species by way of mobile genetic elements such as pathogenicity islands, transposons, and plasmids (17). Knowledge of the existence of E. coli-associated Shiga toxin and its role in human health emerged in 1983, when a study by Karmali et al (18), discovered a substance acutely lethal to African green monkey kidney (Vero) cells in the filtered stools of children suffering HUS that was antigenically indistinct from the toxin produced by Shigella dysenteriae. This study was published in the same year as another investigation by Riley et al (19), examining the stools of patients involved in outbreaks of HC, where an unusual serotype of E. coli, expressing the somatic (O) antigen 157 and the flagellar (H) antigen 7, was implicated as the causative organism (20). Subsequent investigations have led to widely accepted associations between the presence of E. coli expressing the Shiga-like toxin and cases of HUS and HC (20), and also revealed a secondary variant of the toxin that could not be inactivated by anti-Shigella toxin antibody. These two variant types of the toxin have come to be referred to as Stx1 (antigenically similar) and Stx2 (antigenically distinct) (20). Although more recent studies have linked diarrhoeal diseases directly to the genes of virulence factors carried by both O157 and non-O157 STEC strains (21, 22), much of the diagnostic efforts of the medical community remain geared towards only the most prominent serotype possessing them; O157:H7.
Figure 1: Pathologies of gastrointestinal pathotypes of *E. coli*, including enterotoxigenic *E. coli* (ETEC; depicted here with heat-labile (LT) and heat stable (ST) enterotoxins), enteroaggregative *E. coli* (EAEC), diffusely-adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC). *E. coli* expressing Shiga toxins (STEC) are often associated with the characteristic EPEC attachment factors intimin and bundle forming pili (BFP), which permit close association to, and microvillus effacement of, intestinal enterocytes. Shiga toxin and the EPEC attachment factors work together synergistically to give an enhanced likelihood of progression to severe haemorrhagic diseases, and this co-interaction forms the basis of the classification enterohaemorrhagic *E. coli* (EHEC).

Figure sourced from Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev. 1998;11(1):142-200.
1.3 Pathology of STEC and the role of intimin (eaeA)

The Shiga toxin variants Stx1 and Stx2 are defined as AB$_5$ multiunit toxins, and are encoded on two mobile genetic elements (H19B and 933W, respectively) referred to as temperate lambda bacteriophages (23). These prophages are composed of double-stranded DNA, typically flanked by functioning insertion sequences, and have the ability to insert themselves into the chromosomal DNA of E. coli through the action of recombinases (24). The prophages display considerable genetic mosaicism (25). These elements permit the host E. coli to express Shiga toxin and, if functionally intact, can also multiply and excise themselves from E. coli genomes through integrases and phage-mediated lysis under conditions that promote lysogenic behaviours (26-28). The toxins expressed by the Stx genes form a protein complex composed of two subunits; a single 30 kDa A subunit attached to five identical 7 kDa non-covalently pentamERICALLY-bound B subunits (15). The B subunit pentamer component of the toxin binds to glycosphingolipid globotriosylceramide (Gb$_3$) receptors of absorptive villi and Paneth cells in the mammalian intestine, and globotetraosylceramide (Gb$_4$) surface receptors in renal glomerular cells and brain endothelia (15, 17, 26, 29). The binding by B subunits promotes the internalisation of the toxin complex, whereupon the A subunit induces apoptosis of the target cell through the depurination of 28S eukaryotic ribosomal RNA, effectively halting all protein synthesis in the endoplasmic reticulum (15, 29). The toxins are able to cause bloody and non-bloody diarrhoea through the killing of intestinal endothelial cells, although there is some speculation as to whether this is through direct killing or indirectly by the toxin inducing mesenteric ischaemia in the regional vasculature (6). The toxins are also able to translocate across the gastrointestinal endothelia by an unresolved transcellular pathway and enter the systemic circulation, from where it is documented to have a host of pathological thrombotic and immunomodulatory effects on both the renal glomerulus and systemic microvasculature (15, 30) A histological section showing Shiga toxin-mediated formation of a renal thrombus is shown in Figure 2. The mechanism by which the toxins traverse the circulation remains a point of conjecture amongst researchers (31), and the systemic effects are not usually accompanied by bacteraemia (30). It should be noted at this point that Stx1 and Stx2 have variable toxicity; Stx2 toxins are noted to have at least a 100-fold higher toxicity against renal endothelial cells than Stx1 (5, 15) due to differences in the DNA sequences encoding the toxin components (32). As a result, Stx2 is the variant
Figure 2. Thrombus (arrow) in an arteriole of renal glomerular tissue as induced by Shiga toxin-mediated killing of Gb3-receptor expressing renal tissue. This histology section is from a fatal case of postdiarrhoeal HUS.

Figure sourced from Tarr PI, Gordon CA, Chandler WL. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. The Lancet. 2005;365(9464):1073-86.
most often associated with clinical cases of STEC and the progression of such cases to the more severe systemic complications of the disease such as HC and HUS (15, 32, 33). Stx2 is also noted to have a number of subvariants (Stx2, stx2c2, stx2d, stx2e, and stx2f) each with varying toxicity (34, 35). Perhaps unsurprisingly, O157 serotypes associated with clinical symptoms are generally found to have very high carriage rates of the Stx2 toxin variant, usually in preference to Stx1, although comparable carriage rates may be seen outside this serogroup (21, 32, 36). The enhanced virulence of serotype O157:H7 may be attributed to accessory virulence factors carried on a highly conserved 92-104 kb nonconjugative F-like plasmid referred to as pO157 (15, 37). pO157 contains over 100 open reading frames encoding putative virulence factors such as proteases, haemolysins and adhesins on a heterogeneous mix of prophages, transposons and genetic elements thought to have evolved to aid intestinal colonisation in cattle (15, 16, 38). The virulence factors encoded on pO157 are obliquely related to O157:H7 infection; HUS-causing strains do not consistently have the full complement of putative virulence factors (39), and the pathogenicity of the plasmid in concert with Shiga toxin is somewhat muddled due to conflicting findings and heterogeneity in the expression of factors among strains (15, 40).

Another notable virulence factor, and one with particular relevance to the pathogenesis of STEC and enteropathogenic E. coli (EPEC), is the adherence factor intimin, also referred to by its DNA encoding sequence eaeA. Intimin is a 94 kDa bacterial outer membrane protein encoded within a 35-42 kb mobile pathogenicity island referred to as the Locus of Enterocyte Effacement (LEE) (5). The LEE itself contains 41 open reading frames, highly conserved in EPEC, that encode a syringe-like type III secretion system, capable of injecting over 50 bacterial effector proteins directly into the cytosol of eukaryotic cells (15, 16). Intimin itself serves as an anchoring ligand on the bacterial cell surface for receptor proteins injected by the secretion system, thereby permitting close association to affected enterocytes (41, 42). These effector proteins, in conjunction with intimin, then permit LEE-positive E. coli to reshape the actin cytoskeleton of intestinal epithelial cells and form characteristic 10 µm pedestal formations on the cell surface, as shown in Figure 3 (41, 43). The LEE mechanism is also responsible for the formation of attaching and effacing (A/E) lesions, a characteristic debriding of microvilli on intestinal epithelial cells (5) (see Figure 3). Both the A/E lesions and pedestal formation are the defining histological characteristics of EPEC, and are thought to effect diarrhoea through
Figure 3. Pedestal formation and A/E lesions of EPEC and EHEC. The actin cytoskeleton remodelling ability of the *E. coli* LEE mechanism manifests as characteristic pedestal formations on the surface of intestinal epithelial cells underlying LEE-carrying *E. coli*. (a) depicts the pedestals as visible by scanning and transmission electron microscopy. (b) depicts normal intestinal microvilli, while (c) shows their characteristic effacement by the LEE mechanism.


Pictures (b) and (c) sourced from Mainil JG, Daube G. Verotoxigenic *Escherichia coli* from animals, humans and foods: who's who? J Appl Microbiol. 2005;98(6):1332-44.
aiding diffuse adherence and motility of LEE-positive bacteria across epithelial surfaces (41, 42, 44). Intimin is not the only adherence factor in EPEC (45), but mutant analysis indicates it is crucial for the optimal function of the LEE mechanism (42, 44), and is regarded as the principal adhesin for the formation of close associations with intestinal epithelial cells and colonisation of the intestine by EPEC (15, 42). Due to the integral role of intimin in the formation of A/E lesions, the presence of the encoding gene, eaeA, acts as a satisfactory marker of the LEE mechanism of EPEC bacteria (45, 46). Although diarrhoea can certainly arise in LEE-negative STEC infection (41), and although the exact pathogenicity also relies on accessory virulence determinants (as shown in Table 1) and host factors (32, 39, 47), the close association formed by the components of the LEE mechanism are recognised to greatly aid in the delivery of Shiga toxins to enterocytes and the underlying mesenteric vasculature in vivo (41). Most of the strains isolated from outbreaks of HC and HUS and those infections with more severe symptoms typically express both Shiga toxin and intimin (21, 33, 36), and are usually designated along with carriage of pO157 as the pathotype enterohaemorrhagic E. coli (EHEC) (39). The tendency for HUS patients to form strong antibody responses to intimin and other LEE components underscores the importance of the adhesion determinant in advanced STEC infections (16).

1.4 Disease course and therapy

Due to the confluence of host and virulence factors, as well as the infecting STEC or EHEC strain and accessory virulence factors present, the clinical spectrum of STEC is appreciably broad and patient outcomes may be difficult to predict. Symptoms can range from asymptomatic or subclinical (and hence unreported) diarrhoea to severe haemorrhagic and neurological complications and death. Clinical cases of STEC infection typically present as self-limiting, with painful abdominal cramps and non-bloody diarrhoea that occur 1-8 days post ingestion (4, 43), but may progress to bloody diarrhoea without concomitant fever or raised leucocyte count after a further 2-7 days (15, 43). The progression to HC and HUS is difficult to predict (27), but is especially frequent in children <10 years of age (43, 48), the immunocompromised and the elderly, with the highest rates associated with EHEC (stx+, eaeA+) and particularly O157 serotypes (15, 43). The rate of progression to HC and HUS is generally 10-15%, subject to patient factors, the accessory virulence factors present, and the Shiga toxin variant
TABLE 1: Accessory virulence factors associated with enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) E. coli pathotypes

<table>
<thead>
<tr>
<th>Pathotype</th>
<th>Element Name</th>
<th>Element type</th>
<th>Insertion site</th>
<th>Key virulence factors (function)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EPEC</strong></td>
<td>LEE</td>
<td>PAI</td>
<td>selC, pheU, pheV, yehV, yecE, ssrA, wrbA, z2577†</td>
<td>T3SS (cytoskeletal reorganisation), Tir (translocated intimin receptor), Map (mitochondrial membrane potential disruption), EspB, EspF, EspG, EspH and EspZ (T3SS effector proteins)</td>
</tr>
<tr>
<td></td>
<td>EAF (pMAR2)</td>
<td>Plasmid</td>
<td>Not applicable</td>
<td>BFP (adhesin)</td>
</tr>
<tr>
<td></td>
<td>PP2</td>
<td>Phage</td>
<td>ybhC_ybhB</td>
<td>EspJ, Cif (blocks mitosis) and NleH</td>
</tr>
<tr>
<td></td>
<td>PP4</td>
<td>Phage</td>
<td>torS_torT</td>
<td>NleD, NleC, NleB and NleG</td>
</tr>
<tr>
<td></td>
<td>PP6</td>
<td>Phage</td>
<td>ompW</td>
<td>NleF, NleH and NleA</td>
</tr>
<tr>
<td></td>
<td>IE2</td>
<td>PAI</td>
<td>serX</td>
<td>NleE and LifA-like</td>
</tr>
<tr>
<td></td>
<td>IE5</td>
<td>PAI</td>
<td>ssrA</td>
<td>EspG and EspC</td>
</tr>
<tr>
<td><strong>EHEC</strong></td>
<td>LEE</td>
<td>PAI</td>
<td>selC</td>
<td>T3SS (cytoskeletal reorganisation), Tir (translocated intimin receptor), Map (mitochondrial membrane potential disruption), EspB, EspF, EspG, EspH and EspZ (T3SS effector proteins)</td>
</tr>
<tr>
<td></td>
<td>pO157</td>
<td>Plasmid</td>
<td>Not applicable</td>
<td>EspP, toxinB, LifA/Efa (lymphocyte adhesion/activation inhibition), StcE (T3SS complement disrupter), HlyA (cell lysis) and EhxA (cell lysis)</td>
</tr>
<tr>
<td></td>
<td>Sp3/CP-933K</td>
<td>Phage</td>
<td>ybhC_ybhB</td>
<td>NleB, NleC, NleD and Cif (blocks mitosis)</td>
</tr>
<tr>
<td></td>
<td>Sp9/CP-933P</td>
<td>Phage</td>
<td>yciE</td>
<td>NleA (also known as EspI), NleF, NleG, EspM, NleH and EspO</td>
</tr>
<tr>
<td></td>
<td>Sp14/CP-933U</td>
<td>Phage</td>
<td>serU</td>
<td>TccP and EspI</td>
</tr>
</tbody>
</table>


PAI: pathogenicity island. LEE: Locus of Enterocyte Effacement. IE: insertion element. PP: prophage. EAF: EPEC adherence factor
expressed (2, 26, 48). HUS itself is characterised by the onset of microangiopathic anaemia (damaged erythrocytes), thrombocytopenia (reduced platelet levels) and may be accompanied in adults by thrombotic thrombocytopenic purpura (TTP; a diffuse formation of microangiopathic thrombi, often with concurrent neurological abnormalities) (20, 49). HUS is the clinical manifestation of Shiga toxin-induced damage of the kidney glomerular vasculature, and may even result in long term sequelae such as renal insufficiency and neurological aberrations (43). Overall, 3-5% of HUS cases are fatal (15). Generally, non-O157 infections have a milder course than that of O157:H7; diarrhoea has a greater likelihood of being bloody (>90% chance with O157 vs 60%), abdominal cramping is usually more severe, and the need for hospitalisation (43% vs 18%) and progression to HUS (10-15% vs <10%) is more pronounced in O157 infection (7). These tendencies can largely be related to the carriage of eae and stx2 virulence factors; stx2 is associated with HUS in both O157 and non-O157 STEC infection. On the other hand, carriage of stx2 is generally less common in non-O157 STEC, usually in favour of the less toxic stx1 (21, 32, 36). O157:H7 and disease causing non-O157 STEC generally have very high carriage rates of eae (36), and combined eae and stx2 carriage is regarded as a stronger predictor for bloody diarrhoea and HUS than the presence of serogroup O157 in multivariate analysis (50). The HC that accompanies severe STEC or EPEC infection can mimic other intestinal diseases such as Crohn’s disease and induce elevated faecal calprotectin levels, while chronic STEC infections may result in irritable bowel syndrome (IBS) (15). Many physicians have an incomplete understanding of STEC infection and do not consider non-O157:H7 infection as part of a differential diagnosis (51). The clinical presentation of STEC infection may also potentially lead to confusion with other gastrointestinal disorders such as intussusception, appendicitis, inflammatory bowel disease (IBD), or infection with Clostridium, Shigella, Yersinia, Salmonella, or Campylobacter species (20, 27, 52, 53).

EPEC infection typically presents as a self-limiting, acute, watery diarrhoea with vomiting and low grade fever, although some instances of protracted EPEC diarrhoea have been described (43). EPEC infection is a significant contributor to infant diarrhoea in developing countries (43), and although comparable prevalences have not been seen in infants of developed nations since the 1960s, genetic profiling indicates EPEC may still be significant to children’s diarrhoea (43, 54).
Treatments options for STEC infections are largely supportive, as antibiotics that induce DNA damage in *E. coli* have a tendency to activate a lysogenic SOS response in the Shiga prophage and cause upregulation of the expression of the Shiga toxin (35). As a result, treatments involving quinolones, ciprofloxacin, in addition to antimotility agents, are contraindicated in STEC infection, having been associated with increased incidences of progression to HUS (15, 30, 55). Parenteral volume expansion with liberal amounts of intravenous fluids (15, 56), particularly in conjunction with peritoneal dialysis is the recommended course for advanced cases of STEC infection (27, 31). Early administration of the monoclonal anti-Shiga toxin antibody eculizumab is also recognised to increase platelet counts and limit toxin-mediated neurological effects (31, 56). The development of vaccines capable of stimulating a strong antibody response against pathogenic *E. coli* antigens such as intimin (35, 57), is currently ongoing, albeit hampered by the difficulty in finding an appropriate animal model (43).

1.5 Epidemiology

The global impact of STEC infection is difficult to estimate because of inconsistencies in reporting methods between countries and the varying prevalences of detected and undetected serotypes within such countries. The most recent estimate, a meta-analysis of papers and databases of 21 countries, places the global number of acute STEC infections at approximately 2.8 million per year, with an estimated 3890 cases of HUS, 270 cases of end-stage renal disease, and 230 mortalities (58). STEC infections are usually sporadic and generally affect children and the elderly, with the typical source of infection being the ingestion of contaminated foodstuffs (43). The natural reservoir of STEC is the intestine and rectum of cattle, and the contamination of processed beef carcasses combined with modern mass distribution is recognised as the classic cause of larger outbreaks (5, 59, 60). STEC may also be carried by a variety of farmyard animals including sheep, goats, chickens, pigs and deer, with outbreaks traced to contact with these animals or consumption of improperly prepared foodstuffs (48, 61, 62). Infection with *stx* EPEC is mostly confined to diarrhoeal sporadic cases in children <2 years of age and outbreaks are usually related to contaminated weaning foods, milk formula and fomite transmission in nurseries and paediatric units (43). The shedding of EPEC in faeces may be asymptomatic in adult carers and older children, and so these are thought to be the natural reservoir of infant EPEC infection (43). An adult form of EPEC
diarrhoea may be observed in instances of an ingestion of a large inoculum and/or perturbed gastric acids (43). EPEC infection is associated with traveller’s diarrhoea in developing countries (43).

In recent years, STEC outbreaks have also been associated with person-to-person spread, as well as contaminated or unpasteurised milk, juice, sprouts and other vegetables (2). The durability of STEC in bovine faecal matter and groundwater is thought to permit the spread of STEC to mass-produced crops via effluent run-off and exposures through environmental water sources (35, 57, 63, 64). Perhaps the most notable case of an outbreak of STEC infection in recent times was the 2011 O104:H4 outbreak in Germany, involving an especially virulent strain of non-O157 E. coli that caused over 4000 STEC illnesses, 908 cases of HUS and 90 deaths (55). The source of infection was ultimately traced to a batch of contaminated fenugreek sprouts (55), and although the strain did not employ intimin as its primary attachment factor (65), the outbreak served to highlight the contribution of non-O157 STEC to human disease in developed countries. Non-O157 STEC infection is usually dominated by the serogroups O26, O111, O145, O103, O45 and O121, which together typically comprise ~70% of the non-O157 STEC infections in many countries and are well represented amongst isolates retrieved from sufferers of HUS, TTP and bloody diarrhoea (32, 36). These ‘big six’ or ‘gang of five’ (some researchers omit O45) possess many of the virulence factors and associations to complicated enteric disease seen in O157:H7 strains (9), although the dominant strain may vary from country to country (see Table 2). The contribution of the ‘big six’ to STEC disease is noted to be underreported (7), and international data suggests that the proportion of STEC disease caused by non-O157 serogroups is increasing (7, 8, 66, 67).

In New Zealand, the annual notification rate for all STEC infection as recorded by ESR has been increasing since 1997, peaking with 4.6 cases per 100,000 in 2013 (203 cases; see Figure 4) (13). The Enteric Reference Laboratory at ESR identified 88.1% of received STEC isolates as being of the serotype O157:H7 in 2014, with the remainder confirmed as non-O157 E. coli (10.2%) or of undetermined serotype, a result analogous to that of the previous year (68). Most STEC cases are unrelated or occur in small outbreaks confined to a family or geographic region with exposure to contaminated farmland or private water supplies as primary risk factors (69). Studies involving serogrouping of New Zealand STEC in cases of human illness indicate New Zealand has usual and
TABLE 2: Prevalent and predominant STEC serogroups by country

<table>
<thead>
<tr>
<th>Country/Continent</th>
<th>Prevalent serogroups</th>
<th>Predominant serogroup (O157 vs non-O157)</th>
</tr>
</thead>
<tbody>
<tr>
<td>United States</td>
<td>O157, O26, O111, O103, O121, O45, O145</td>
<td>O157</td>
</tr>
<tr>
<td>Canada</td>
<td>O157, O55, O125, O26, O126, O128, O18</td>
<td>O157</td>
</tr>
<tr>
<td>South America</td>
<td>O1, O2, O15, O25, O26, O49, O92, O11</td>
<td>Non-O157</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>O1, O2, O15, O25, O26, O49, O92, O11</td>
<td>O157</td>
</tr>
<tr>
<td>Continental Europe</td>
<td>O157, O26, O111, O104, O103, O128, O91, O113, O2, O9, O145</td>
<td>Both</td>
</tr>
<tr>
<td>Australia</td>
<td>O157, O111, O26</td>
<td>Both</td>
</tr>
<tr>
<td>Japan</td>
<td>O157, O26, O111</td>
<td>O157</td>
</tr>
</tbody>
</table>

Figure 4. Number of all STEC infections reported to ESR by community and hospital based diagnostic laboratories 1997-2014. The number of notifications of STEC infections has been increasing since 1997, with typically 90% of cases recognised to be caused by *E. coli* O157:H7. Graph sourced from Notifiable diseases in New Zealand: Annual Surveillance Report. The Institute of Environmental Science and Research, 2014. Available at: https://surv.esr.cri.nz/surveillance/annual_surveillance.php
unusual non-O157 serogroups (O26, O84, O103, O123, O176, and O180), likely related to agricultural practice (70-72). Due to deficiencies in current diagnostic approaches in STEC detection, it is entirely plausible that these or other serotypes may have an unappreciated role in diarrhoeal disease in New Zealand.

1.5 Diagnostic techniques

*E. coli* O157:H7 is currently a notifiable disease in New Zealand, and in accordance with recommendations by the CDC (2), *E. coli* isolates presumptively identified as O157:H7 are sent by hospital and community diagnostic laboratories to the Institute of Environmental Sciences and Research (ESR; a Crown Research Institute) for confirmation as STEC, serotyping and inclusion in monthly and annual Public Health Surveillance reports (such as those available at [https://surv.esr.cri.nz/surveillance/surveillance.php](https://surv.esr.cri.nz/surveillance/surveillance.php)). Like many diagnostic laboratories in the United States and Europe (53, 67, 73), the isolation of O157:H7 by New Zealand laboratories is typically based on the selective and differential culture of faecal samples on Sorbitol-MacConkey Agar, supplemented with cefixime and potassium tellurite (CT-SMAC; as available from Fort Richard Laboratories in New Zealand). The vast majority of *E. coli* O157:H7 strains, unlike most commensal *E. coli* and ~95% of enteric bacteria (43, 74), are unable to ferment the carbohydrate sorbitol within 24 hours, and the lack of fermentation by such colonies will typically render them colourless in the presence of agar pH indicators (6) (see Figure 5). SMAC-based plating of faeces is appropriate for the differentiation of O157:H7, but has a number of limitations. The first and perhaps most important shortcoming is that the majority of non-O157 *E. coli* serotypes are sorbitol-fermenting (8), and so potentially these Shiga-toxigenic *E. coli* would not be discriminated from other enteric bacteria with use of this medium, nor sufficiently reported in national surveillance datasets. When considered alongside international and domestic trends citing increases in prevalence of non-O157 and all STEC infection, and considering the role of non-O157 within the New Zealand environment, the need for a prevalence study to determine the contribution of non-O157 to diarrhoeal disease is evident. This shortcoming should be considered in conjunction with the fact that the pathogenicity of certain strains of non-O157 is comparable to that of O157:H7. Furthermore, sorbitol-fermenting variants of O157 do exist, most notably strains of
Figure 5. SMAC agar. Among enteric bacteria, *E. coli* O157:H7 are near-uniquely unable to ferment the carbohydrate sorbitol within 24 hours, and so the use of SMAC-based media has become a popular screening method for O157:H7. Sorbitol non-fermenting colonies typically appear colourless (arrow) with the incorporation of Neutral Red as a pH indicator.
O157:H- (also referred to as O157:NM, or non-motile); a serotype of considerable pathogenicity encountered in Germany that is also tellurite sensitive (74, 75).

The diagnostic algorithm in use at Southern Community Laboratories (SCL) at Dunedin Hospital is to perform confirmatory identification of sorbitol non-fermenting colonies with use of matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) and latex agglutination with O157 antibody analysis before sending live specimens of any O157 or sorbitol non-fermenting E. coli to ESR (76). The diagnostic algorithm is represented diagrammatically in Figure 6. This algorithm effectively precludes confusion with other enteric bacteria that may not ferment sorbitol (ie. Aeromonas) (73), or have antigens that cross-react with O157-specific antibodies (ie. Citrobacter freundii, Escherichia hermannii), but misses sorbitol-fermenting E. coli of any serotype (48). The rapid clearance of detectable STEC bacteria from the gastrointestinal tract (7-12 days after onset of diarrhoea, even in cases of HUS (77, 78)), the action of antibiotic regimens in-situ in faeces potentially inhibiting plate-based growth (78) and the fact that samples are usually taken several days after the onset of diarrhoea can conspire to limit detection of O157:H7 strains (27, 49, 77). Moreover, freeze-, pH-, or salt-stressed E. coli (as may be encountered in faecal samples that are kept on ice for live transport) are recognised to have compromised resistance to normally harmless selective agents such as cefixime and tellurite on plate media (79-81).

An attractive alternative to the use of plate-based culturing are PCR-based assays, and many studies in recent years involving primer sequences capable of the amplification of virulence genes specific to E. coli have been successfully developed (46, 82, 83). PCR assays of processed faecal samples promise potentially the most sensitive, specific and economical method for the large-scale detection of pathogenic E. coli (83-85), and one configurable for the simultaneous detection of multiple E. coli virulence determinants (14, 86, 87). As PCR-based techniques are based on the multiplication of select specific genetic sequences within a sample, the technique is an alternative to culture-based techniques that hinge on the growth of isolates amongst other enteric bacteria. The use of quantitative real-time PCR (qRT-PCR) assay platforms, such as the Life Technologies Viia™ 7 Real-Time PCR System, can potentially provide quantitative information on pathogenic E. coli without need for post-PCR processing (88). Pre-enrichment of faecal samples is recommended for PCR protocols established for the diagnosis of STEC.
All diarrhoeic stools are plated to CT-SMAC for overnight culture

Limitations
- Cannot isolate Sorbitol non-fermenting *E. coli*
- Contingent on *E. coli* growth
- Not as sensitive as molecular methods

Sorbitol non-fermenting colonies

MALDI-TOF
(confirms *E. coli* or *Shigella*)

Latex agglutination
(monoclonal O157 antibodies)

Discarded

Sent to ESR

**Figure 6.** STEC testing algorithm for diarrhoeal stools received by Southern Community Laboratories, Dunedin Hospital. This culture-based algorithm detects the *E. coli* serotype O157:H7 through sorbitol non-fermentation, MALDI-TOF identification and latex agglutination with monoclonal anti-O157 antibodies. Sorbitol non-fermenting *E. coli* isolates suspected of being O157:H7 are sent to ESR for confirmation and serotyping.
infections (56). The more rapid turn-around time for qRT-PCR can also allow for more rapid diagnoses of STEC infections, informing timely and accurate treatment (56), avoiding contraindicated antibiotics or invasive procedures, and permitting the early adoption of control measures in outbreak situations (2, 43). qRT-PCR reactions may also be configured so that multiple assays occur within the same reaction mix simultaneously, in a process referred to as multiplexing. Another avenue for PCR investigation is the relatively novel loop-mediated isothermal amplification (LAMP) technique (89). LAMP employs an alternative amplification mechanism to amplify target sequences, and has proven to be of use in diagnostic settings (90, 91). While LAMP is not amenable to multiplexing, the technique could be explored as a form of rapid, inexpensive screening of O157 STEC. There are currently no diagnostic measures in place for the screening of EPEC at Dunedin Hospital.

1.6 Aims and Study Objectives

The primary aim of the project was to develop and test a multiplex qPCR assay suitable for the detection of STEC and/or intimin carrying E. coli and to use this assay to determine the prevalence of these bacteria in diarrhoeic stools sourced from SCL, Dunedin Hospital. A secondary aim of the project was to develop the assay to be appropriate for use in a diagnostic setting, through maximising sensitivity of the test, adapting the assay for high-throughput testing, and simplifying pre-PCR extraction methods. A tertiary objective was to examine the prevalence of STEC or intimin-carrying E. coli in faecal samples set aside for prospective H. pylori or inflammatory bowel disease (IBD) testing at SCL, Dunedin Hospital.
Project Aims/Objectives

1. To develop a qPCR assay or assays capable of detecting STEC and EPEC in faecal samples
   - Validate a (preferably) multiplex PCR assay suitable for the simultaneous detection of STEC and intimin-carrying *E. coli*
   - Determine if the assay can be used to provide relevant quantitative information on STEC and/or intimin-carrying *E. coli* from patient samples
   - Use the assay to determine the prevalence of STEC and intimin-carrying *E. coli* in diarrhoeal stools sourced from Dunedin Hospital

2. Adapt the diagnostic method to be suitable for use in routine medical laboratory testing
   - Evaluate the efficacy of a selection of DNA extraction techniques, and decide upon which would be most appropriate for large-scale screening of faecal diarrhoeal stools
   - Evaluate the effect of pre-enrichment on the sensitivity of the PCR assay to detect STEC or intimin-carrying *E. coli* within faecal samples with use of the selected DNA extraction method
   - Adapt assay conditions and the use of reagents to be as economical as is feasible
   - Examine the viability of a LAMP-based PCR assay to classify O157 serogroups

3. Use the assay to survey prevalences of STEC and EPEC in diarrhoeal stool samples set aside for *H. pylori* or IBD testing in Dunedin Hospital
2 Diarrhoeagenic STEC and EPEC growth characteristics

2.1 Introduction

The detection of STEC through multiplex PCR assays has been well established in the scientific literature, with many studies in the past two decades detailing the efficacy of PCR to deduce the presence of \textit{E. coli}-specific virulence determinants in a variety of mammalian faecal samples including human (14, 46, 86, 87, 92-95). The data derived from these and other studies has contributed greatly to the understanding of the epidemiology of STEC, but many diagnostic laboratories, even in developed countries, have yet to implement PCR-based technologies (2, 10, 96). For many laboratories, the cost of PCR reagents and specialist training is the main factor in deciding diagnostic algorithms, but the varying sensitivities of PCR based technologies must also be taken into consideration. The limit of detection of sorbitol-fermenting STEC on CT-SMAC based culture of naturally infected human faeces has been assessed to be $1 \times 10^4$ – $1 \times 10^6$ CFU/g (97, 98). There is some variability in the limits of detection of STEC and other bacteria in faecal samples by various qRT-PCR methods (the limit of detection ranges from $3 \times 10^2$ to $1 \times 10^5$ CFU/g faeces) (87, 95, 99-101), and the sensitivity can be influenced by the amount of endogenous \textit{E. coli} in faeces (95, 102). The low numbers of STEC during infection and the rapid clearance of the organism from the intestine necessitates highly sensitive testing for accurate reporting (73, 98, 103). Pre-enrichment of faecal samples in non-selective broth is recommended as a practical approach to boost the sensitivity of PCR assays (56, 73). Pre-enrichment is thought to have the dual benefits of increasing resident STEC populations beyond the limits of PCR-based detection whilst diluting the effect of faecal compounds that would normally be inhibitory to Taq polymerase (56, 104). Although the growth dynamics of \textit{E. coli} in liquid media is arguably one the most well established concepts in microbiological research, studies on the dynamics of STEC culture growth in short incubation (<6 hours) is sparse.

The following experiments were undertaken with the intent to evaluate the most appropriate liquid broth medium for the growth of a representative range of STEC strains in a timespan appropriate for use in a day-to-day diagnostic setting.
2.2 Materials and Methods

2.2.1 Culture media
The growth media for cultivation of the bacterial strains used in this study were sourced in dehydrated form, prepared according to the manufacturer’s instructions and autoclaved at 121°C for 15 minutes. The broths used were Lennox LB (Invitrogen) and agar (Bacto™, BD Diagnostics), EC broth (Fort Richard Laboratories), Trypticase Soy Broth (TSB) and agar (TSA) (BD Diagnostics), and Brain Heart Infusion (BHI) reductant (BD Diagnostics).

2.2.2 Bacterial strains
2.2.2.1 Standard Operating Procedure for STEC containment
In accordance with the Department of Microbiology and Immunology Health and Safety policies and PC2 provisions, a Standard Operating Procedure (SOP) for the appropriate handling and disposal of STEC-containing culture material was devised (see Appendix 1).

2.2.2.2 E. coli O91:H-, O130:H11 and O84:HNM
*E. coli* reference strains O91:H- (*stx*1⁺), O130:H11 (*stx*2⁺) and O84:HNM (*eae*⁺, *stx*1⁺) used in the study were obtained from the Enteric Reference Laboratory, The Institute of Environmental Sciences and Research (ESR; Wellington) (see Table 3). Initially, strains carrying only one of the *stx* variants under investigation were chosen on the basis of safety, although subsequent testing revealed O84:HNM to be also *stx*2⁺.

2.2.2.3 E. coli O111:K88:H- and O157:H-
*E. coli* reference serotype O111:K88:H- (*eae*⁺) was obtained from Dr. H Brooks’ culture collection (Dept. Microbiology and Immunology, University of Otago), and a *stx* O157:H- strain was obtained from Dr. Michelle McConnell’s culture collection (Dept. Microbiology and Immunology, University of Otago). See Table 3 for strain reference information.

2.2.3 Storage and Culture
*E. coli* O91:H-, O130:H11, and O84:HNM were resuscitated from lyophilised ESR stock cultures by resuspension in TSB and application to TSA for overnight incubation at 37°C under aerobic conditions. *E. coli* O111:K88:H- and O157:H- were received in skim milk
### TABLE 3: BACTERIAL STRAIN INFORMATION

<table>
<thead>
<tr>
<th>Serotype</th>
<th>NZRM</th>
<th>ESR ERL No.†</th>
<th>Ref No.</th>
<th>eae</th>
<th>stx1</th>
<th>stx2</th>
<th>Isolate source</th>
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<tbody>
<tr>
<td>O91:H-</td>
<td>4152</td>
<td>ERL97-3929</td>
<td>CDC 97/30206</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Ovine (Meat)</td>
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<td>ERL02-1805</td>
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<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Human</td>
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<td>O111:K88:H-</td>
<td>735</td>
<td>-</td>
<td>NCTC 911</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>Infant NEC*</td>
</tr>
<tr>
<td>O157:H7</td>
<td>3647</td>
<td>-</td>
<td>-</td>
<td>(found to be positive)</td>
<td>Negative</td>
<td>Negative</td>
<td>Infant NEC*</td>
</tr>
</tbody>
</table>

*NEC = Necrotising Enterocolitis  †Enteric Reference Laboratory, Environmental Sciences and Research
stored at -80°C, resuspended in TSB, incubated overnight at 37°C and plated on TSA. After Gram staining, the strains were stored in Microbank (ProLab Diagnostics) bead stocks at -20°C. Bacteria used for the Optical Density (OD) curve, broth comparison and qPCR extraction experiments were plated on TSB from these bead stocks and incubated at 37°C overnight under aerobic conditions. Due to the propensity of STEC to lose stx-bearing prophages through multiple culture passages, passaging of more than five derivatives of a bead stock was avoided.

2.2.4 Gram staining of bacterial strains
All of the *E. coli* strains were Gram stained and examined under high magnification (x100 oil lens) before culture and qRT-PCR work to confirm the identity of the bacterial growth and check for possible contaminants. Culture plates were examined to ensure consistent colony morphology.

2.2.5 Standard OD curve
OD standard curves of strains O91:H-, O130:H11, O111:K88:H- and O84:HNM were prepared to provide a fast and reliable means to determine Colony Forming Units (CFU) mL⁻¹ from optical density measurements.

2.2.5.1 Turbimetric measurement (Optical density)
Growth from single colonies of each of the *E. coli* strains given above were suspended separately in 10 mL of TSB broth and cultured under atmospheric conditions at 37°C for 18 hours. The broth cultures were diluted 2-fold in sterile TSB diluent and 0.1 mL of each dilution was dispensed into a 96-well flat bottom tissue culture plate (Falcon®) in triplicate. Using sterile TSB as the blank, the optical density of the dilutions was read by spectrophotometric absorbance at 600nm with a Varioskan plate reader (Thermo Scientific Research Edition, using SkanIt software v2.4.5.9). Any values that were negative after normalising against the blank density were disregarded in subsequent calculations.

2.2.5.2 Viable Bacterial Count
The overnight broths were diluted 10-fold in sterile PBS and 0.1 mL of the 10⁻⁵, 10⁻⁶, and 10⁻⁷ dilutions were dispensed in triplicate to TSA plates and incubated overnight under aerobic conditions at 37°C. After overnight incubation, the bacterial colonies on the plates
with a colony count between 20-250 colonies were counted, and the average of the triplicate cultures was recorded. An uninoculated culture was included with all cultures and subsequently plated to check for broth contamination.

2.2.5.3 **Standard Curve**
A standard curve was created for each of the bacterial strains by plotting the absorbance obtained from each dilution against the CFU mL$^{-1}$ obtained from the bacterial count using the Microsoft 2013 Excel software program.

2.2.6 **Enrichment Broth Comparison**
2.2.6.1 **Turbimetric Measurement (Optical density)**
To determine the optimal enrichment broth for short-phase *E. coli* culture conditions, an overnight culture of each of the *E. coli* strains (O111:K88:H-, O91:H-, O130:H11 and O84:HNM) was diluted to 2x10$^3$ CFU mL$^{-1}$ in each of the documented sterile broths (BHI, EC, LB and TSB) based on optical density measurements as described above. This inoculant (1 mL) was dispensed to two sets of 20 mL Universal bottles, each containing 9 mL of each of the four broths. One set was incubated under aerobic conditions for 3 hours after a thorough mixing by pipette aspiration. The culture (0.1 mL) was dispensed to 3 wells of a 96-well plate and the absorbance at 600nm was measured against an equivalent uninoculated blank incubated alongside the inoculated cultures. The other set was incubated for 6 hours and subjected to the same measurement. Each of the strains were subjected to this experiment twice, such that 2 x 3 hour OD$_{600nm}$ readings and 2 x 6 hour OD$_{600nm}$ readings, each in triplicate, were obtained. One of the two cultures with O111:K88:H- was inoculated with 2 x 10$^4$ CFU of the bacterium. Using standard curves for each of the strains, the absolute CFU mL$^{-1}$ of the incubated cultures was calculated and graphed using Microsoft Excel 2013 and ANOVA analysis (with Tukey’s Multiple Comparisons post-test) GraphPad Prism (v5.03) software.
2.3 Results

2.3.1 Standard OD\textsubscript{600nm} curve for \textit{Escherichia coli} strains used
The OD\textsubscript{600nm} readings and CFU calculated from plate readings for each of the \textit{E. coli} strains are expressed graphically in Figure 7. The resultant equations (eg. \( y = 2E x -10x +0.0071 \) for \textit{E. coli} stain O111:K88:H-) were used as a basis of calculating CFU mL\textsuperscript{-1} from broth culture ODs in subsequent experiments.

2.3.2 Broth comparison experiment results
The growth of the \textit{E. coli} strains O111:K88:H-, O91:H-, O130:H11 and O84:HN0M after six hours of incubation at 37°C under aerobic conditions is shown in Figure 8. The cultures that were incubated for 3 hours did not return OD\textsubscript{600nm} values when normalised against their respective blanks (sterile broth) and so were not included. The 6 hour values showed considerable variability in the growth of the different \textit{E. coli} strains in the broths, and also in the comparative performance of the broths across the strains. EC broth was found to have the poorest growth overall, with OD\textsubscript{600nm} readings so low as to be indistinguishable from the uninoculated broth control after 6 hours of incubation for strains O91:H-, O130:H11 and O111:K88:H- (when the O111:K88:H- cultures were inoculated with the standard amount (2 x 10\textsuperscript{3} CFU mL\textsuperscript{-1}) of bacteria). The only instance of this broth appearing to perform better than any other occurred during testing with O84:HN0M, however the differences in mean calculated CFU with that of BHI were not judged statistically significant (\( p <0.05 \)) by ANOVA analysis. The broth that appeared to provide the best growth overall was BHI, however this appeared to be heavily strain dependent, with comparatively poor growth in this broth by O84:HN0M (BHI growth was significantly lower than TSB (\( p <0.05 \)) and considerably lower than LB (\( p <0.001 \)). Although TSB had significantly lower growth (\( p <0.001 \)) than BHI for O111:K88:H- when using the higher inoculant (2 x 10\textsuperscript{4} CFU mL\textsuperscript{-1}), this broth was not statistically lower than BHI for any of the other strains, and did not display the variability in comparative growth as seen with LB. One of the broth comparisons conducted for O111:K88:H- used a higher inoculum, and is expressed separately in Figure 8.
Figure 7. Standard CFU-OD curves. The standard curves shown here are based on the mean Optical Densities (600 nm) of 2-fold dilutions of each of the Escherichia coli strains shown here plotted against the mean CFU mL$^{-1}$ as calculated from triplicate plate viable counts of each of the undiluted cultures.
Figure 8. Comparison of growth of the listed serotypes of *Escherichia coli* over 6 hours at 37°C, aerobic conditions. The broths shown are Brain Heart Infusion (BHI), *E. coli* broth (EC), Lennox Luria Broth (LB) and Tryptic Soy Broth (TSB). All broths were inoculated with $2 \times 10^4$ CFU ($2 \times 10^3$ CFU mL$^{-1}$ of broth) of the *E. coli* strains shown, except those depicted in the O111:K88:H- #2 graph, which were inoculated with $2 \times 10^5$ CFU ($2 \times 10^4$ CFU mL$^{-1}$). The error bars depict the standard error of the mean (SEM) with the graphs of O111:K88:H- each composed of triplicate OD reads. The other graphs represent two separate tests, like that of O111:K88:H11, but with the data merged into one graph as the growth conditions were exactly the same. Any values that returned negative after normalisation against a sterile blank were excluded. The results show considerable variability in the performance of the broths across the *E. coli* strains tested. EC broth provides the least optimal conditions, with what growth that is able to be detected by OD$_{600nm}$ usually significantly less ($p < 0.05$) than the growth seen in the other broths. Although BHI and LB did permit effective growth of the *E. coli* strains shown, the growth appeared to be highly dependent on the strain and inoculation amount, TSB was judged to be the most consistent supporter of short phase growth across the *E. coli* strains tested and therefore the most suitable for the general culture of *E. coli* strains likely to be encountered in faecal specimens.
2.4 Discussion

The intent of the broth comparison experiment was to determine which commonly used non-selective broth would be most suitable for the pre-enrichment growth of STEC. TSB was found to be the best for growth of the STEC reference strains over a 6 hour incubation, with EC broth performing poorly compared to the other broths. The experiment assessed a shorter incubation time that could be incorporated within a daily diagnostic schedule. Previous research has indicated this minimises overgrowth of non-pathogenic *E. coli*, preventing STEC detection through metabolic crowding of the media (105). One of the complications in STEC research, aside from the low numbers of pathogenic strains during active infection (103), is the effect of selective agents. The choice of non-selective media was based on the findings of Rocha *et al* (106) and Fukushima *et al* (105), who conducted testing of STEC strains in antibiotic-supplemented media. The addition of antibiotics such as vancomycin, cefsulodin and novobiocin does improve the selectivity of enrichment broths for *E. coli* over that of other enteric bacteria for longer incubations (105), but are demonstrated to constrain the growth of *E. coli* in incubations of 6 hours or less (106). Instances of injury or stress, as may be encountered in faecal environments, are recognised to predispose pathogenic *E. coli* strains to the action of selective agents (73, 107, 108). There is also some evidence of novobiocin, cefsulodin and vancomycin causing selective inhibition of certain strains of STEC, such as strains of O91 and O111 serogroups (109). TSB and EC broths were selected for comparison on account of their ubiquity; a review conducted by Vimont *et al* (110) concluded TSB and EC were the most frequently used broth for the isolation of STEC, a fact corroborated by many instances of their use in other studies (56, 62, 64, 85, 103, 105-109, 111-116). BHI and LB were readily accessible to the researcher and were included in comparisons on the basis of their amenability to short-phase incubation, with demonstrable examples of these broths fostering increases of STEC populations from environmental sources within 2 hours of incubation (93, 104, 109). EC was noted by Rocha *et al* to have impeded growth in comparison to the other assessed broths, but he placed emphasis on a ciprofloxacin additive as being a root cause (106). In considering the restrained growth of STEC within EC broth, it was concluded that the media was not wholly non-selective; a later review of EC constituents revealed bile salts (as an agent selective for enteric bacteria) and that this likely retarded the short-term growth of STEC in the absence of ciprofloxacin (81, 106). In considering the applicability of pre-
enrichment procedures to a diagnostic setting, it was decided to base the use of TSB for 3 hour incubations by interpolating the 6 hour results of the comparative broth experiment as the plate reader could not detect changes in broth OD at 3 hours. In retrospect, this decision should have been supported with comparative plate counts before and after 3 hours TSB incubation similar to that conducted later in the faecal spiking experiments. Incubations of 2-3 hours to ‘resuscitate’ enteric bacteria has precedents in the literature (73, 92, 108), and data from Fukushima et al and Rocha et al supported the use of 3 hour TSB culture to boost STEC CFU even in the presence of other Gram negative bacteria likely to be encountered in faecal matrices (105, 106). Immunomagnetic beads, despite their use in improving sensitivity (98), are not generally adopted by diagnostic facilities on account of cost and labour requirements (104). The use of buffered peptone water for the resuscitation of injured STEC and the use of 42°C incubations for better selectivity for *E. coli* among other enteric bacteria remain avenues for future pre-enrichment investigations (105, 107, 117).
3 Detection of STEC and EPEC in faecal samples with use of qPCR

3.1 Introduction

One of the frontiers in modern diagnostic research is the adoption of molecular-based techniques for use in the diagnosis of infectious disease, with many areas of research focussed on testing the validity of PCR-based assays for the detection of STEC (12, 86, 87, 92, 95, 114, 118-121), and comparing these with existing culture-, immunological-, and biochemical-based techniques employed in clinical laboratories (56, 98, 99, 114, 122). PCR is based on the extracellular amplification of customisable DNA sequences under thermocycling conditions, and the technique can be tailored to detect specific genetic markers independent of host cell viability. Currently, the diagnosis of STEC in diagnostic laboratories centres on the characteristic inability of one particularly virulent serotype, O157:H7, to ferment sorbitol, with no phenotypic markers available to discriminate other sorbitol-fermenting serogroups of STEC from non-pathogenic E. coli (43, 82). PCR-based assays, then, could be employed in diagnostic laboratories to circumvent the restrictions imposed by culture-based techniques and match diagnosis directly to the presence of E. coli virulence factors known to have causative roles in STEC disease (22, 36). qRT-PCR assays are the latest iteration of PCR technology, and offer the potential to detect DNA in the femtogram range (123). Like conventional PCR, qRT-PCR is based on the amplification of specific DNA fragments, but engineered to produce increased fluorescence in proportion to the amplified DNA sequence through the use of fluorescent hydrolysis probes or DNA-intercalating dyes such as SYBR green (for an explanation of qRT-PCR with use of SYBR green or hydrolysis probes like those used by TaqMan, see Figure 9) (88). The name ‘real-time’ refers to the continuous monitoring of fluorescence by qRT-PCR platforms during thermocycling; providing the amplification efficiency of the assay is suitable, the cycle threshold value (the point at which the fluorescence is deemed to be statistically significant) should be inversely proportionate to the amount of the target sequence (46, 88, 123). In this way, qRT-PCR can also provide quantitative information on a DNA sequence within a sample, and potentially provide information of clinical importance (88). Multiple primer and probe sets can be combined within a single reaction, and so there exists the potential to develop
**Figure 9.** Principles of RT-PCR methods using SYBR Green and hydrolysis probes. SYBR Green acts as an intercalating dye that greatly increases in fluorescence upon intercalating with double stranded DNA, and so can be used as a real-time marker of DNA amplification as the target sequence amplifies. Hydrolysis probes (such as those utilised by TaqMan) are conjugated to a fluorescent emitter (such as 6-carboxyfluorescein (FAM-reporter) and a quencher fluorochrome (the emitter and quencher are depicted here as blue and red circles, respectively). The proximity of the quencher and emitter on either end of the probe oligonucleotide supresses the fluorescence of the emitter even when the oligonucleotide anneals to the complementary sequence. As the action of Taq polymerase during extension phases will hydrolyse the probe oligonucleotide, fluorescence will increase with each thermocycle as more emitter molecules are separated from quencher molecules.

Figure adapted from van der Velde VHJ, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJM. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: Principles, approaches, and laboratory aspects. Leukemia. 2003;17:1013-34.
a single assay capable of simultaneously detecting the *E. coli* virulence factors *eae, stx1* and *stx2* within faecal samples (14, 82).

One of the obstacles to the use of PCR-based methodologies is PCR inhibition. As PCR relies upon the action of DNA-polymerases to multiply DNA sequences, and as there exist many biological compounds that prevent polymerases from working properly, the process of DNA extraction, or the removal of DNA from tissues and from the influence of inhibitors, is particularly important (103, 124). One of the gold standards amongst DNA extraction techniques is the phenol-chloroform technique, which involves centrifugation-mediated organic phase separation of DNA from cellular salts and proteins. Although the technique is prevalent in research applications for its ability to remove a wide range of PCR inhibitors, the toxicity of phenol and the labour-intensity of the protocol limits its diagnostic practicality.

Another avenue of interest is loop-mediated isothermal amplification or LAMP, a novel PCR technique based on the amplification of DNA fragments at a constant temperature of 55-65°C through the use of six special looping primers and a DNA polymerase with a primer-displacing ability (89). The technique has seen some adoption in diagnostic settings (a LAMP platform is used to detect *Clostridium difficile* infections at Dunedin Hospital) (76), and the samples to be harvested in this project present an ideal opportunity to use the technique to distinguish O157 serogroups.

The intent of the following experiments was to validate and develop a practical multiplex RT-PCR technique suitable for the detection of the *E. coli* genes *eae, stx1* and *stx2* (along with *uidA* as a marker of all *E. coli*), and to apply this in conjunction with an optimal pre-enrichment broth to screen faecal samples sourced from Dunedin Hospital. The initial focus of testing was a comparison of the efficiency of four DNA extraction methods to determine which would be the most appropriate for the processing of faecal samples to be screened, with consideration for diagnostic applicability.

A key focus in the experiments before the collection and screening of the main faecal samples was to determine the limit of sensitivity of the assay once the optimal pre-enrichment broth and extraction had been selected. The test was performed on faecal
samples spiked with known concentrations of STEC in conjunction with a performance test of the pre-enrichment broth to encourage the growth of STEC with a faecal matrix.

Attempts were made, in the interests of minimising PCR reagents costs and labour, to adapt the RT-PCR screening of the processed faecal samples to 384-well qPCR plates and to automate the loading process with the use of a robotic sample-loading platform.

After the processed faecal samples were screened, a number of experiments were performed to investigate the role of PCR inhibition in a number of suspect samples.
3.2 Materials and Methods

3.2.1 Probes, primers, buffers, and qPCR mix

The sequences of four sets of custom probes and primers designed to amplify sections of the *E. coli* genes *uidA*, *eae*, *stx1*, and *stx2* were obtained from sequences pretested by Nielsen *et al* (2003) (46). The *stx2* primers were designed by Nielsen *et al* to detect all *stx2* subvariants with the exception of *stx2e*, which does not have a significant role in human health. All of the primers used in the project were tested by Nielsen *et al* against GenBank sequences to ensure no ‘irrelevant’ sequences were amplified, and subsequently tested against a panel of enteric bacteria for specificity to *E. coli* by Anklam *et al* (82). The probes and primers themselves were obtained from Biosearch Technologies using the Custom Oligonucleotide Synthesis service (for sequence information, refer to Tables 4 and 5). The lyophilised probes and primers were individually resuspended in TE buffer (10mM TRIS buffer, 1mM EDTA, pH 7.9) to concentrations of 100 µM, from which working stocks of 20 µM were derived. The forward and reverse primers for each gene were combined in pairs in a joint 20 µM working solution. All 20 µM primer and probe stock aliquots were kept at -4°C with the probes kept in foil to prevent UV degradation of the fluorescent dyes. Stocks (100 µM) were stored frozen for longer periods at -20°C. The qPCR reaction mix used for all reactions was PerfeCTa® qPCR ToughMix™ (2X reaction mix; Quanta Biosciences). The *uidA* probe and primer set (amplifying an *E. coli*-specific region of the β-glucoronidase gene) were included in later multiplex assays as an effective internal amplification control and an indicator for any non Shiga-toxigenic or intimin-carrying *E. coli* in faecal testing (82).

3.2.2 General PCR assay conditions

For all assays prior to the main testing of faecal samples for the prevalence study, a master mix composed of ToughMix, and the probes and primers as required by each of the PCR-based investigations was designed and distributed to wells of a 96-well reaction plate (MicroAmp® Fast Optical Reaction Plate, Applied Biosystems®). For each assay, the master mix was tailored such that each well reaction would total 20 µL, composed of 10 µL (2X) ToughMix, 600 nM of each required forward and reverse primer, 200 nM of each required probe, and 1 µL of extracted DNA, with the remaining reaction volume made up with TE buffer. qPCR reactions were carried out with one of two available
### TABLE 4: Primer sequences

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<th>Oligonucleotide</th>
<th>Oligonucleotide sequence (5′-3′)</th>
<th>Annealing temperature (°C)</th>
<th>Amplified fragment length (bp)</th>
<th>Gene NCBI GenBank Ref. No.</th>
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<td>59</td>
<td>Z11541.1</td>
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<tr>
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<td>stx1 forward primer</td>
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<td>X65949.1</td>
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† Sequence modified from source paper (Anklam et al., 2012 (82)) using Primer Express software (Applied Biosystems; V2.0) for more appropriate annealing temperature

### TABLE 5: Probe sequences

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<tr>
<th>Oligonucleotide</th>
<th>Oligonucleotide sequence (incl. 3′, 5′ modifications)</th>
<th>Annealing temperature (°C)</th>
<th>Fluorescent Tag Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>uidA probe</td>
<td>5′d FAM-TCGCGGATCCGGTGGCAGTGGCAGT-BHQ-1 3′</td>
<td>70</td>
<td>495 nm</td>
</tr>
<tr>
<td>eae probe</td>
<td>5′d Quasar 670-ATAGTCTCGGATCCGGCGACCAATACC-BHQ-2 3′</td>
<td>69</td>
<td>670 nm</td>
</tr>
<tr>
<td>stx1 probe</td>
<td>5′d CAL Fluor Orange 560-TCCAGAGGAAAGGCGGCTATACC-BHQ-1 3′</td>
<td>70</td>
<td>560 nm</td>
</tr>
<tr>
<td>stx2 probe</td>
<td>5d 5-TAMRA-ATGTCTATCCGGCGGCTTGGTACCATCTT-BHQ-2 3′</td>
<td>69</td>
<td>555 nm</td>
</tr>
</tbody>
</table>
thermocyclers; an Applied Biosystems™ Viia7 Real-Time PCR system (Life Technologies) utilising Applied Biosystems Viia7 software (v1.2.4), or an Applied Biosystems 7500 Fast Real-Time PCR system (Life Technologies) using 7500 software (v2.0.6). The PCR cycling parameters for reactions performed on the Viia7 system were as follows: a singular holding step of 50°C for 2 minutes, then a singular holding step of 95°C for 10 minutes, followed by a cycling phase of 95°C for 15 seconds and 60°C for 1 minute, with these two cycling temperatures repeated at these intervals for 40 cycles. The thermocycler used for each assay will be noted accordingly in each methodology section below.

3.2.3 DNA Extraction Methods
To remove substances from broth-based and faecal-based culture material potentially inhibitory to the action of DNA polymerase, four established DNA extraction methods were adapted.

3.2.3.1 Boiling extraction
The boiling extraction used is an adaption from boiling methods documented by Malorney et al and Walsh et al (125, 126). For any culture tested with this method, 1 mL of culture was dispensed to a sterile 1.5 mL microcentrifuge tube and centrifuged for 10 minutes at 18,000 x g, before resuspension in 100 µL TE buffer (filter-sterilised with a 0.22 µm filter). Autoclave tape was placed over the lid of the microcentrifuge tube, a heated syringe was used to puncture the lid of the microcentrifuge tube and the container was boiled in a pot cooker for 10 minutes. The sample was then cooled on ice before a final centrifugation step of 13,000 x g for 5 minutes. The supernatant from this final centrifugation was used as template DNA.

3.2.3.2 PrepMan™ extraction
The PrepMan™ (Applied Biosystems) kit based extraction was performed as per the manufacturer’s instructions with minor modifications to the volumes used. For the culture prepared with this method, 0.2 mL was dispensed to a 2 mL microcentrifuge tube and the sample was centrifuged for 3 minutes at 16,000 x g. The supernatant was discarded and 200 µL of the PrepMan Ultra Sample Preparation Reagent was added to the tube and the tube mixed on a vortex mixer for 10-30 seconds. The sample was then
heated in a pot cooker for 10 minutes at 100°C and centrifuged for 3 minutes at 16,000 x g, with the supernatant used as template DNA.

3.2.3.3 Phenol-chloroform extraction

The phenol-chloroform method documented here is adapted from Sambrook et al (127), with initial washing steps derived from Giraffa et al (128), centrifugation speeds based on recommendations from Zoetendal et al (129), and alcohol precipitation steps as described by Hoff-Olsen et al (130). For each sample extracted by the phenol-chloroform method, 1 mL of culture was washed twice by centrifugation at 12,000 x g and resuspended in 1 mL PBS or TE buffer. The sample was then centrifuged at 12,000 x g before resuspension in 500 µL of a TE extraction buffer (10mM TRIS, 2.5mM EDTA, 0.5% SDS, 100mM NaCl, 100 µg mL⁻¹ proteinase K, pH 7.9). The resuspended sample was then incubated in the TE extraction buffer for 1-2 hours in a 56°C water bath before the addition of 500 µL of 25:24:1 phenol-chloroform-isoamyl alcohol solution (Sigma-Aldrich®) to each sample tube. Each sample was forcefully inverted by hand until a milky emulsion formed. Each sample was then centrifuged at 14,000 x g for 5 minutes. The aqueous upper layer of the separated phases was extracted by pipette and transferred to a new microcentrifuge tube, taking care to avoid drawing up the proteinaceous interface or the lower organic phase. The sample was then mixed with 500 µL of pure chloroform with a repeat of the centrifugation and phase separation of the phenol-chloroform step. The DNA of each sample was precipitated adding NaCl to a concentration of 0.2M based on the net weight of the sample and mixing by gentle pipette aspiration. Chilled ethanol (100%; 1 mL) was added to each precipitate and the mixture was centrifuged at 14,000 x g before removal of the ethanol supernatant by pipette. This ethanol washing step was repeated with 1 mL of 70% ethanol with each sample before the samples were left open in a fume hood for 30 minutes to facilitate evaporation of the last of the ethanol. Each sample was resuspended in 30 µL of RNAse-free water except where documented. Extracted samples were kept at -4°C or -20°C for longer storage.

3.2.3.4 Chelex-100 extraction

The Chelex-100 method described is adapted from the method as described by Malorney et al (125). For each sample processed using this method, 1 mL of culture material was transferred to a 1.5 mL capped microcentrifuge tube and centrifuged at 10,000 x g for 10 minutes. The supernatant was discarded and the pelleted material was resuspended in 300
µL of a 6% weight-to-volume suspension of Chelex-100 resin in Milli-Q (filtered, de-ionised) water. The suspension was incubated at 56°C in a water bath for 15-20 minutes before vortex mixing for 5 seconds and a further incubation at 100°C for 8 minutes on a heating block. Each sample was then chilled on ice for 2 minutes and centrifuged at 14,000 x g for 5 minutes, with the supernatant from this final centrifugation used as template DNA.

3.2.4 PCR validation assays and amplification efficiency check

To ensure that the PCR probes, primers, PCR mix and thermocycler conditions selected were capable of reproducible and efficient DNA amplification of the target sequences, an overnight 37°C TSB culture of each of the E. coli strains O111:K88:H-, O91:H-, and O130:H11 was processed with the phenol chloroform method as described above. The concentration of the extracted DNA was determined through UV spectroscopy (DNA-50 function with NanoDrop 1000 v3.7.1, Thermo-Fisher Scientific), and diluted with TE buffer based on these readings to a concentration of 1 ng µL⁻¹. Each of these samples was diluted serially ten-fold in TE to give a 5-order-of-magnitude log₁₀ standard of template DNA for each of the gene assays. Four individual PCR assays were set up using the standard PCR conditions (outlined above) for the Applied Biosystems Viia7 thermocycler. Using the log₁₀ standard of each of the DNA templates and the standard PCR conditions (outlined above), a standard curve was constructed for each of the genes. Each of the assays had a custom master mix containing only the probe and primer set of the genes each E. coli strain was expected to contain (a uidA and eae assay with an O111:K88:H- log₁₀ DNA standard, an stx1 assay with an O91:H- DNA log₁₀ standard, and an stx2 assay with an O130:H11 log₁₀ DNA standard). Each well of each assay had 1 µL of each of the log₁₀ dilutions of DNA template added and each dilution was assayed in triplicate. A well to which no template DNA was added was included with each assay to act as a negative amplification control. A cursory check of eaeA, stx1 and stx2 specificity was also performed at this time; each of the primer sets was tested individually with STEC strains mentioned in this section that lacked the corresponding gene to rule out non-specific or off-target amplification.

To ensure that the target sequence of each assay was amplifying efficiently, the Applied Biosystems Viia7 software was used with the data of the log₁₀ DNA standards mentioned
above to calculate a standard curve based on the cycle threshold (Ct) values of each of the assays, from which the amplification efficiency could be calculated.

3.2.4.1 PCR amplicon size check
To verify that the primers described in Table 4 were amplifying the correct sequence fragments as predicted by Primer Express software, each of the probe and primers sets of the multiplex were prepared in separate PCR mixtures and used to amplify the predicted sequences from a phenol-chloroform extracted overnight TSB culture of O84:HNM. The DNA extracted (5 µL) by each assay was combined with 5 µL of bromophenol blue loading dye and run on an electrophoretic 2% agarose gel (SeaKemp® LE agarose, Lonza) with ethidium bromide added at a concentration of 50 µg mL⁻¹. The DNA samples were run alongside molecular ladders and a negative control composed of a post-PCR mix with no added template.

3.2.4.2 Multiplex amplification efficiency check
A comparative amplification efficiency check was completed to ensure that none of the individual assay amplification efficiencies would be significantly affected by multiplexing. Using new master mixes containing combinations of each of the probe and primer pairs, a DNA standard was constructed using standard PCR conditions under the same conditions used for the initial PCR validation. The samples’ dilutions were distributed in triplicate in the same manner as the individual assays, but instead of only adding one DNA type, 1 µL of each of the O111:K88:H-, O91:H-, O130:H11 dilutions were added to each reaction well (the master mix had a reduced amount of TE buffer so that each well volume would still sum to 20 µL) so as to provide a DNA template for all of the assays in the multiplex. The probe and primer sets were tested sequentially, adding eae, stx1, and stx2 to a uidA reaction in separate assays to see if any one reaction interfered notably with the others. The software of the Applied Biosystems Viia7 was then used to generate a standard curve for each of the assays within the complete multiplex reaction. The reaction efficiencies were then compared to the reaction efficiencies of the individual assays. A software-generated standard curve for each of the assays within the multiplex can be viewed in the results section. As with the individual assays, wells with all of the reaction components but no template were included as amplification negative controls.
3.2.5 PCR extraction method yield comparison

3.2.5.1 Relative DNA yield by cycle threshold values

To compare the efficiency of the extraction methods in extracting DNA, a TSB culture of O111:K88:H- was incubated at 37°C for 6 hours and extracted by each of the extraction methods detailed above (boiling, PrepMan, phenol-chloroform and Chelex-100). To ensure consistency, each of the extraction methods was conducted on the same O111:K88:H- culture which was kept chilled on ice (4°C) between each extraction. As the PrepMan method involved a smaller amount of culture to be processed compared to the other DNA extraction methods used (0.2 mL as opposed to 1 mL), appropriate dilutions of each of the extracted samples were carried out in TE buffer to account for the unequal volume extracted. The extracted material was applied to a qRT-PCR assay in triplicate wells with a master mix composed of only the uidA probe and primer set. The cycle threshold (Ct) values were then compared across the samples that had been processed by each of the four extraction methods to determine the most efficient form of DNA extraction. An uninoculated culture was processed with the four extraction methods alongside the live culture and included in the subsequent qRT-PCR assay as a functioning amplification control. A comparison of DNA yield by direct DNA spectroscopy was avoided as some proteinaceous compounds not removed by Chelex-100 were recognised to interfere with such readings (131).

3.2.5.2 Gel photos of extracted DNA

To examine the physical state of the extracted template DNA generated by the four extraction methods, an O91:H- TSB culture incubated for 6 hours was extracted by the boiling method and Chelex-100 method, while another O91:H- TSB culture was similarly incubated for 6 hours was extracted by the phenol-chloroform and PrepMan methods. The DNA extracted (5 µL) by each method was combined with 5 µL of bromophenol blue loading dye and run on an electrophoretic 0.7% agarose gel (SeaKemp® LE agarose, Lonza) with ethidium bromide added at a concentration of 0.5 µg mL⁻¹. The DNA samples were run alongside molecular ladders and negative controls composed of uninoculated TSB cultures that were processed alongside each of the extracted cultures. TRIS pH 7.0 was used as a running buffer.
3.2.6 PCR-based quantitation

To accurately estimate the number of STEC CFU in the prepared standards for use in determining multiplex sensitivity and possibly the CFU mL\(^{-1}\) of faecal specimens, a survey of the genome sizes of \(\text{stx}^+\) and \(\text{stx}\) \(E.\ coli\) was conducted using the \(E.\ coli\) Genome Assembly and Annotation Report table on the NCBI database (Available at [http://www.ncbi.nlm.nih.gov/genome/167](http://www.ncbi.nlm.nih.gov/genome/167) under the tab labelled “Genome Assembly and Annotation Report”). Using the table feature to sort all of the catalogued sequences by ‘level’ (how complete the archived sequence was), all of the available complete \(E.\ coli\) FASTA sequences were imported and analysed using pDRAW software (pDRAW32 1.0, revision 1.1.121; ACACLONE software, available at [http://www.acaclone.com](http://www.acaclone.com)). Using the “find sequence” function, a search was run on the four probe sequences (as featured in Table 4) to distinguish STEC genomes from non-STEC genomes and to enumerate copy numbers of each sequence. Each sequence search permitted up to 3 base pair mismatches. The genome sizes (as recorded on the Genome Assembly and Annotation Report table) for STEC and a sample of non-STEC were then separately pooled and graphed using PRISM software, from which the average and median could be calculated. Calculations based on the molecular weight of DNA molecules in conjunction with the mean STEC genome size were used to arrive at a representative weight per STEC genome. With the weight of a representative STEC genome known it was possible to relate the number of genomes by weight to the concentration of DNA in an extracted sample (assessed as ng \(\mu\text{L}^{-1}\) through NanoDrop readings of phenol-chloroform extracted \(E.\ coli\) cultures) through to Ct values when a known concentration of DNA was amplified. A standard curve of estimated numbers of genomes per well could be then constructed and used on qRT-PCR plates to estimate the number of STEC genomes in other wells.

3.2.7 Use of faecal specimens

In accordance with the New Zealand Human Tissues Act 2008 and the New Zealand Health Information Privacy Code 1994, a protocol was devised to source the faeces and pertinent personal information regarding the patients involved in a manner that would not infringe upon the patient’s right to privacy of health information. In brief, faecal samples that satisfied the selection criteria sourced from the health agency (SCL) were to be collected and labelled with a unique identifier (SCL’s internal identification number) and with the donor’s age status (a binary status of ≥12 years of age or younger)
and whether the donor was registered as an inpatient or outpatient at the time of collection. Only this information was to be provided by SCL staff, with no other personal information provided to the researcher. Ethics committee approval was obtained through designing the collection of faecal samples so as to comply with exception (e)i of the Health Information Privacy Code 1994 Rule 10 (Limits on the use of health information) which states “[health records may be used] for statistical or approved research purposes provided [the] information is not published in a form that could reasonably be expected to identify the individual”. The acquisition of informed consent of the patients from whom the samples were sourced was deemed unnecessary on the basis of the Human Tissues Act 2008 Section 20 (e), which states informed consent is not required for collection or use of “the carrying out, by using for a secondary purpose tissue that is a body or is collected from a living individual or a body, […] of research that has received the approval of an ethics committee […]”. An explication of other relevant sections of the Code and the Act as provided on request of further clarification to the University Ethics Committee can be found in Appendix 2. For a flowchart detailing the collection of samples that was also provided to the Ethics Committee, see Figure 10.

3.2.7.1 University of Otago Ethics Committee Approval

In compliance with the University of Otago Human Ethics Committee’s policies on the acquisition and use of human tissue(s) for research, an application for Human Ethics Committee (Health) Departmental Approval of Projects using Health Information was completed (see Appendix 3 for a copy of this document and a copy of SCL approval) prior to work with faecal specimens commencing. For documents relevant to University of Otago Ethics Committee and their approval, please refer to Appendix 2.

3.2.7.2 Maori Affairs Consultation

In agreement with the New Zealand Human Tissue Act 2008 Section 18 and the University’s policy regarding consultation with Māori, a discussion with the Ngāi Tahu Consultation Committee was completed to ensure the project was compliant with Tikaka (“best practice”; a concept which encompasses respect, collection, retention, and return of the body parts or genetic material of Māori, usually on spiritual grounds). The use of faeces in the manner proposed for the project was deemed appropriate by the committee as Māori did not desire return or especial retention of faecal material or derivatives on spiritual grounds. A copy of the Ngāi Tahu Consultation Committee document can be
SCL continuously receives stool samples from community and hospital.

Samples defined as diarrhoeic (stools that take the shape of the container) are selected.

SCL staff use each sample to inoculate an individual enrichment broth provided by the researchers. The containers are only labelled with age, in/out patient status and a unique ID assigned by SCL.

The researcher obtains the inoculated enrichment broths and incubates to encourage the growth of bacteria.

The culture is processed to extract and purify the DNA for use with molecular assays.

Extracted DNA is frozen for long term storage.

Once enough extracted DNA is amassed, all of the extracted DNA to date is processed together in a large batch. This assay only examines bacterial (STEC) DNA.

After a defined time period (likely to be a 3 month catchment period) the data will be compiled by the researcher and used to make conclusions as to the prevalence of the organism in the samples.

At the conclusion of the study, the data will be shared with SCL and it is expected SCL will determine whether the assay and/or data is of retrospective clinical or diagnostic relevance in combination with patient information that only they are privy to.
found in Appendix 4, along with the pertinent sections of the Southern District Health Board’s Tikaka Best Practice document.

3.2.7.3  **Containment, handling and disposal**
A SOP was researched and developed to ensure proper conduct, including the use of Personal Protection Equipment (PPE) and containment facilities. A copy of this document is included in Appendix 1 and was incorporated as a physical copy with the University safety documentation in the laboratory.

3.2.7.4  **Selection criteria and storage of live specimens**
As there remains no standardised definition of diarrhoea, diarrhoeal stool specimens were selected from amongst the samples submitted to SCL on the basis of visual sample viscosity. Samples of a loose consistency that were noted to ‘take the shape’ of their parent container under their own weight were regarded as diarrhoeic and included in the study.

As some experimentation with faecal material required the storage and retention of live specimens, approval to store live specimens on site in a -80°C freezer in cryobroth (Fort Richard laboratories) was obtained from SCL.

3.2.8  **Stationary phase O84:HNM growth curve**
For qPCR assay sensitivity testing, an 18 hour growth curve of O84:HNM was carried out to determine when this strain reached stationary phase. The intent of the growth curve was to ensure that reasonably accurate CFU mL⁻¹ dilutions could be made from stationary phase cultures but without the discrepancy between OD₆₀₀nm reads and CFU mL⁻¹ that was likely to occur in the strain’s death phase. For this experiment, two 20 mL Universal bottles containing 10 mL of TSB were inoculated from a single colony of O84:HNM grown on TSA overnight at 37°C. The cultures were well mixed, and the OD₆₀₀nm of these were read using 100 µL in triplicate wells of a 96-well microtitre plate with the Varioskan plate reader. These two Universal bottles were incubated in a 37°C incubator under aerobic conditions. One of these Universal bottles was mixed by pipette aspiration and 100 µL of culture was dispensed for OD₆₀₀nm reads in triplicate on the hour for 9 hours, and then three hourly until 18 hours. The second Universal bottle was dispensed and read in the same manner with mixing only every 3 hours. The OD₆₀₀nm reads, normalised
against an averaged TSB blank, were charted using Microsoft Excel 2013 and Prism software. This protocol also provided information on the growth dynamics of a Universal bottle E. coli monoculture with and without hourly agitation.

3.2.9 Preliminary Faecal screening
To ensure that faecal specimens to be used in subsequent faecal spiking assays did not have endogenous STEC, several diarrhoeic specimens were selected at SCL and 1 mL of faeces was used to inoculate 9 mL of TSB, with 16 mL of faeces frozen at SCL at -80°C in 4 mL of cryobroth (Cryo Broth, Fort Richard Laboratories). The TSB culture was taken back to the University laboratory and 1 mL was extracted with Chelex-100 and subjected to the general PCR conditions on the Applied Biosystems 7500 thermocycler described above. Samples that tested negative for any of the pathogenic genes were used for spiking experiments as outlined below. As with all assays involving faecal specimens, reaction wells with no template were included as negative controls.

3.2.10 Multiplex sensitivity testing by faecal spiking
To determine the limit of sensitivity of the multiplex to detect each of the pathogenic (eae, stx1, stx2) genes in a faecal specimen, and the changes to faecal STEC populations rendered by 3 hours of incubation in nonselective TSB, a protocol to grow and spike faecal samples was developed. An overnight culture of E. coli O84:HNM was grown in TSB at 37°C and the CFU mL⁻¹ determined through OD₆₀₀nm reads. Once calculated, the culture was diluted in sterile TSB to a concentration of 2 x 10⁵ CFU mL⁻¹. This culture was then serially diluted ten-fold in sterile broth to give a dilution series down to (theoretically) 2 CFU mL⁻¹. An unspiked control was included. Two faecal samples (referred to as β and γ) that had tested negative for endogenous STEC were thawed from -80°C and 1.25 mL (to compensate for the dilution in cryobroth) was distributed to multiple 20 mL Universal bottles containing 7.75 mL of sterile TSB. The O84:HNM dilution series (1 mL of each) was then added to spike the faecal samples. The spiked samples were well mixed and 1 mL of the spiked mixture was extracted by the Chelex-100 method (a 0-hour sample), while the remainder of the spiked samples were incubated for 3 hours at 37°C under aerobic conditions. The samples were again mixed, and another 1 mL of the same cultures were then extracted by the Chelex-100 method. The extracted specimens were then tested in triplicate using the complete multiplex under the general
PCR conditions on the 7500 thermocycler alongside a phenol-chloroform extracted O84:HNM DNA standard.

A similar protocol using the same faecal samples (β and γ) and the same spiking method was also completed, with the differences being that the samples were spiked with an exponential-phase O84:HNM culture (incubated for only 6 hours) and each sample was extracted with both the phenol-chloroform and Chelex-100 extraction methods with no incubation phase. The extracted DNA samples were tested with the multiplex PCR assay in duplicate on the 7500 thermocycler under general PCR conditions alongside a $5 \log_{10}$ phenol-chloroform extracted O84:HNM DNA standard. This protocol was intended to investigate any differential effects of growth phase or extraction method in comparison to the protocol above.

3.2.11 Enumeration of spike replicate

To better understand growth characteristics seen in the faecal spiking experiment, the experiment was replicated without added faeces (see section 3.3.7). A O84:HNM TSB culture was incubated for 17 hours at $37^\circ$C, enumerated by OD$_{600nm}$ readings, and diluted to $2 \times 10^5$ CFU mL$^{-1}$ and diluted ten-fold as in the spiking experiment. A 0.1 mL volume of each spike diluted was plated to TSA (effectively a 0-hour sample) and incubated at $37^\circ$C overnight under aerobic conditions. The spiked broths were incubated for 3 hours, mixed, and then 0.1 mL volume was plated to TSA and incubated overnight at $37^\circ$C under aerobic conditions. For both sets of plates, a ten-fold and one-hundred-fold dilution of the $10^5$ and $10^4$ spikes and the broths incubated for 3 hours with the $10^5$ and $10^4$ spikes were plated, in case of innumerable colony counts. The spike and post-incubation broth were enumerated by colony counts on the TSA plates by colony counts of plates that had between 20-250 colonies. The intent of this experiment was to check whether the *E. coli* O84:HNM growth curve was accurate during the strain’s stationary phase and to provide information on the growth of the strain during 3 hours of incubation in the absence of faeces.

3.2.12 Plate counts, NanoDrop readings and calculated Ct values comparison

To check for potential discrepancies between NanoDrop readings, genome estimates calculated by Ct values, and CFU plate counts, 3 x 10 mL TSB cultures were inoculated with O130:H11, grown for 6 hours under aerobic conditions and 1 mL subjected to
phenol-chloroform based DNA extraction. The same cultures were also serially diluted ten-fold in sterile PBS and 0.1 mL of a $1 \times 10^{-7}$ dilution of the culture was plated to TSA and incubated overnight at 37°C under aerobic conditions for plate counting. Plates with between 20-250 colonies were used to generate CFU estimates. The CFU mL$^{-1}$ as calculated from the plate counts, the UV spectroscopy readings, and the genome estimates of the extracted DNA as calculated from Ct values derived from the Applied Biosystems Viia7 thermocycler were compared.

3.2.13 ROX-inclusive master mix validation
In light of a recurring algorithmic correction problem with some assays involving the *eae* probe and primer set, a test utilising an alternate PCR mix (TaqMan Universal Master Mix II with UNG, AB Biosystems) was constructed. As the general mix was also a 2X solution, the experiment simply called for the substitution of ToughMix with Universal Master Mix in the same multiplex and thermocycler parameters. ROX-correction is a default setting on Applied Biosystems Viia7 and 7500 software. The wavelength for the endogenous dye was 610 nm. This experiment was completed on the Viia7 thermocycler, using a phenol-chloroform extracted faecal sample that was found to have had endogenous levels of *stx1* + and/or *stx2* + bacteria in a previous test, and while likely negative for *eae*, was typical of an anomalous pattern of *eae* fluorescence sometimes read by the Viia7. As well as testing whether endogenous dye normalisation would correct the anomalous reading, the test would also be able to demonstrate that the dye could work without impeding the multiplex reaction when testing a faecal-derived specimen.

3.2.14 Collection and processing of faecal samples to be used in main prevalence assay
The faecal specimens (n = 522) used in the main assay were collected from the Microbiology section of SCL, Dunedin Hospital, from the 3rd of July 2014 to the 7th of August 2014. The specimens were all of the samples tested by SCL for common faecal pathogens (refer to Table 6 for common faecal pathogens tested for at SCL, Dunedin Hospital) on each day of the period and which met the selection criteria for diarrhoeic stools. Each day’s worth of specimens was collected for the project 24 hours after SCL testing (live faecal specimens were retained on site for up to a week after SCL testing). Samples collected by SCL staff over the weekends were collected for the project on the ensuing Monday. A 20 mL Universal bottle containing 9 mL of TSB was inoculated with
TABLE 6: Faecal microbiology tests conducted at SCL, Dunedin Hospital

<table>
<thead>
<tr>
<th>Bacteria</th>
<th><em>Salmonella, Shigella, Campylobacter, Yersinia spp, O157:H7, Clostridium difficile, Aeromonas spp, Pleisomonas spp</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasites</td>
<td><em>Giardia, Cryptosporidium, Dientamoeba fragilis, Blastocystis hominis, Isospora or Cycospora cysts</em></td>
</tr>
<tr>
<td>Viruses</td>
<td>Rotavirus</td>
</tr>
<tr>
<td>Special test requests</td>
<td>Faecal calprotectin, <em>H. pylori</em></td>
</tr>
</tbody>
</table>

Faecal panel testing may also include checks for cells, cysts and nematodes under microscopy as indicated.
1 mL of each faecal sample on site, and the approved information (patient age category, in patient status) for each sample was accessed by SCL staff. The date at which SCL had received each sample was also recorded. Each of the samples were extracted from the SCL testing container using 1 mL pipette with tips that had the end of the tips sliced off with a hot razor to widen the bore. Each sample was well stirred before removal, and an estimate of volume was made for samples from which less, or much less than 1 mL of faecal material could be obtained. The inoculated TSB cultures were kept on ice (-4°C) during transportation to the University laboratory, where they were immediately incubated for 3 hours at 37°C under aerobic conditions. After incubation, 1 mL of each faecal culture was extracted using the Chelex-100 DNA extraction method and frozen at -20°C in 1.5 mL microcentrifuge tubes. For the duration of the collection period, other faecal samples (n = 50) that were specifically tested for the presence of *H. pylori* (but separate from the main battery of tests) and that met the selection criteria were also collected. Samples scheduled for *H. pylori* testing were held at SCL and tested weekly on a Thursday, and so a week’s worth of *H. pylori* samples were collected by the researcher on a Friday. On the 31st of July 2014, the researcher noted faecal specimens that were scheduled for faecal calprotectin assays at SCL could also be of relevance to the project, and so specimens scheduled for faecal calprotectin assays from this date that met the inclusion criteria (n = 26) were also included. As the final day of the collection period was a Friday, effectively two weeks’ worth of samples scheduled for faecal calprotectin assays were collected.

### 3.2.15 384-well plate validation assay

To make the testing of the faecal DNA as economical as possible with the use of a 384-well robot plate loader, an assay to ensure that the multiplex could be successfully downscaled to 10 μL-well 384 well qPCR assay plates and still be detectable on the Viia7 thermocycler was performed. Custom master mixes retaining the optimal probe and primer concentrations and 1 μL of extracted DNA sample but with reduced ToughMix (5 μL in each 10 μL well as opposed to 10 μL in each 20 μL well on a 96-well plate) and water volume (volume summed to 9 μL as opposed to 19 μL) were composed and assayed on a 384-well qPCR plate using the Viia7 thermocycler.
3.2.16 Main faecal assays

3.2.16.1 Robot-Loaded 384 plate assays

To load the 384 well plate, a VERSA Aurora 110 Workstation (Appl. 110; Aurora BioMed Inc.) was used utilising v1.0.67 software on a desktop running Windows XP. To have the robotic platform load the 384-well plate, it was necessary to thaw and transfer 50 µL of each of the faecal extracts from the microcentrifuge tubes in which they were stored at -20°C to empty 96-well plates that could be inserted on the platform. An initial test of the robot loader with optimised conditions (optimised primer concentrations of 800 nM for uidA, 600 nM for eae, stx1 and stx2 primers, and optimised probe concentrations of 200 nM for uidA, 300 nM for eae, and 120 nM for both stx1 and stx2 probes), an O84:HNM DNA standard and select faecal samples as a test of function was first completed. For the subsequent large scale testing of the faecal samples for the prevalence study, two 96-well plates, one containing 96 samples and another containing 89 samples plus a 7 point DNA standard (containing 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng µL$^{-1}$ phenol-chloroform extracted O84:HNM) were loaded on the platform. The robot loader was programmed to distribute 9 µL of a pre-made master mix suited to 384 well assays to each well of the 384-well plate, followed by 1 µL of each of the extracted DNAs to two wells each of the 384 well plate along with the standard. Due to the large volume to be dispensed, master mixes with an extra 10% of required volume were used to prevent any discrepancies in pipette tip dispensation leading to a shortfall in master mix. The programme called for a 200 µL tip to be used to dispense the master mix and a tip change was programmed to occur after half of the wells on the plate were filled with master mix. The extracted DNA samples were dispensed using 20 µL pipette tips with a tip change between each sample. The loaded 384 well plate was then assayed on the Viia7 thermocycler using the standard cycling conditions with the reaction volume set to 10 µL on the Viia7 software. The results required a repeat of the protocol and loading programme with a limited number of samples. A troubleshooting follow up test was then completed which involved programming the robot to dispense 9 µL of a viscous solution (glycerol added to water at the approximate viscosity of ToughMix) in a similar manner to a 384-well plate followed by adding 1 µL of bromophenol blue dye as a test of robot dispensation. A reattempt of the first 384-well assay with most of the same DNA samples and standard as above, but with slightly different properties and protocol was then completed. In this attempt, the master mix was made with an extra 15% volume on top of projected well volume requirements. The loading protocol was adjusted to increase
the number of 200 µL pipette tip changes during master mix dispensation to 5 per plate, and to change the amount loaded to 8 µL per well. During the sample loading stage, the amount of sample to be dispensed was adjusted to 3 µL and a mixing step consisting of three short aspirations in the master mix in each well for each sample was added.

3.2.16 Validation of reduced volume 96-well assay

Due to problems with robot loading, and in an attempt to reduce reagent cost of the main assay, an assay to validate further assays of faecal samples using 96-well plates with a reduced volume of ToughMix was completed on the Viia7 thermocycler. This assay was like the 96-well assays that had preceded experimentation with the 384-well plates, but with half the amount of reagents used per well; 5 µL of ToughMix, the optimised probe and primer concentrations, 1 µL of select DNA samples (extracted O84:HNM and select DNA samples from amongst the faecal extractions) and TE to sum the reaction volume to 10 µL per well.

3.2.16.3 Remainder of main assay testing

The remainder of untested faecal DNA samples and samples for which the assays had failed in the second attempt of the 384-well format were assayed in duplicate over 13 x 96-well qPCR plates with the optimised probe and primer concentrations (although non-optimal concentrations of 200 nM were used for the uidA probe, 600 nM for the eae primer and 300 nM for the eae probe), reduced reagent volumes (10 µL) and with standard cycling conditions on the 7500 thermocycler (it should be noted it was the intent of the researcher to use the Viia7 thermocycler for all of the main assays for consistency, but the Viia7 was out of service following the 384-well experimentation). Each of the 96-well plates had 8 wells reserved for positive controls in duplicate and two wells to which no DNA was added to function as a negative control. The positive controls consisted of four wells of phenol-chloroform extracted O84:HNM (two wells with 1 ng and two wells with 0.1 ng), and three “clinical” controls over six wells (two wells with a faecal sample that had tested stx1+ , stx2+ in previous testing (denoted sample #36), two wells dedicated to a eae+ faecal sample (sample #89), and two wells to a clinical negative or uidA+ only faecal sample (sample #50)). Overall prevalences of stx+ and eae+ E. coli amongst the samples processed were computed with 95% confidence intervals (CI) by Stata software (StataCorp. 2013. Stata Statistical Software: Release 13.1 College Station, TX: StataCorp LP).
3.2.17 PVPP column testing

3.2.17.1 Initial test of PVPP processing of faecal samples and BSA assay

Due to some of the results in the main assay having no or poor amplification of the *uidA* gene, initial testing with polyvinylpolypyrrolidone (PVPP) columns to determine if this was a practicable solution to eliminate potential inhibition in the samples was completed using selected samples from the assays. The PVPP columns (Zymo-Spin™ IV-HRC Microcentrifuge tubes; Catalogue no. C1010-50) were used according to an adaption of the manufacturer’s instructions provided in step 11 of the ZR fecal DNA MiniPrep™ Instruction manual provided by Zymo Research. The PVPP columns were prepared by breaking off the tabs below the column and centrifuging the incumbent aqueous solution into a collection tube at 9,000 × g for 3 minutes. For each of the (four) selected samples, 50 µL was deposited into the columns, the caps reapplied and centrifuged again at 9,000 × g for 2 minutes into clean 1.5 mL microcentrifuge tubes. The processed samples were then tested with only the *uidA* probe and primer set in duplicate using the Viia7 with standard cycling conditions and concentrations (200 nM of probe and 800 nM of primer) with reduced (10 µL) well volumes on a 96-well plate. The processed samples were tested alongside the same DNA samples before PVPP processing to see if there was any apparent improvement rendered in fluorescence or Ct value. A separate master mix identical to the others but incorporating 0.1mg mL⁻¹ bovine serum albumin (BSA) was also included on the same plate. A similar test with a 96-well Silicon-A-HRC purification plate (Zymo Research; Catalogue no. C2009) was completed to determine if this item could be used to PVPP-process multiple samples efficiently, rather than to purchase and use an individual PVPP column (Catalogue no. C1010-50) for each sample. For the 96-well Silicon-A-HRC plate protocol, a method based on steps 9 and 10 of the online protocol for the product (available from http://www.zymoresearch.com/dna/microbial-environmental-dna-isolation-1/soil-fecal-plant-dna/zr-96-fecal-dna-kit under the ‘Protocol’ tab) was adapted for use with the sample. For each of the samples to be tested (a selection of prevalence study samples and O84:HNM phenol-chloroform extracted DNA), the foil cover of the wells on a Silicon plate were removed, and 100 µL of TE buffer were added to each well. The Silicon plate was placed over a catchment 96-well plate and the assembly was centrifuged at 1, 470 x g for 5 minutes. An error with the protocol occurred where the buffer that was used to elute the Silicon plate was not removed from the collection plate beneath before 50 µL of the samples to be processed were added to the Silicon plate and the assembly
centrifuged at 2, 250 x g for 6 minutes. It was decided to proceed with DNA amplification as the elution TE buffer was not expected to inhibit the reaction. The processed samples were assayed on the Viia7 thermocycler under standard conditions in a 384-well qPCR plate.

3.2.17.2 Main PVPP processing and retesting of questionable samples
All of the samples suspected of potential inhibition, that is, those that had either no amplification of one or both of their duplicate assays or returned very high Ct values were processed with PVPP columns and retested with the 7500 thermocycler. The PVPP columns (Catalogue no. C1010-50) were prepared as in initial testing and a portion (50 µL) of the samples that showed poor amplification were centrifuged at 9,000 x g for 2 minutes. These samples were then processed using the standard conditions and optimised probe and primer concentrations on the 7500 thermocycler with 1 µL of processed template DNA. The Ct values were compared against the values attained for each sample during the main assays to determine if and how many samples had putatively inhibitory substances removed by the PVPP processing.

3.2.17.3 Internal amplification testing of selected PVPP-processed samples
Due to many amplification results showing little change post PVPP processing, it was decided to conduct an internal amplification assay of selected post PVPP-processing samples to determine if the poor results seen were a result of inhibitory substances that may not have been successfully removed by PVPP processing. For this assay, a selection of samples that showed poor amplification and a slight degradation of uidA Ct with PVPP processing (samples #321, #322, #331, #333), samples that had no amplification whatsoever and no change with PVPP processing (#362, #369, #376) and samples that showed low amplification with no change with PVPP processing (#485, #504) were assayed on the 7500 thermocycler with standard (reduced volume) conditions with an internal amplification plasmid (pChook IAC). The processed samples were to be tested against a negative control without DNA added, to examine whether there was any discernible inhibition remaining after PVPP processing. The assay also included portions of each of the same DNA samples not yet processed with PVPP columns to putatively examine whether there was any change in inhibition seen with PVPP processing. The samples were tested alongside extracted DNA retained from the faecal spiking
experiments functioning as positive controls, and wells with no template added were included as amplification controls.

3.2.18 LAMP based detection of O157 serotypes
An OptiGene Loop Mediated Isothermal Amplification (LAMP) assay was used to distinguish between O157 and non-O157 serogroups amongst the samples found to be positive in the prevalence study. The assay was also tested with a number of samples with dubious positivity (usually very high Ct values of one of the pathogenic genes seen in one of the duplicate wells of a sample). This LAMP assay called for the use of an alternate master mix (Isothermal Master Mix, OptiGene Ltd.) and alternate primers (F3 and B3, LoopF and LoopB, FIP and BIP) intended to target the O157 specific gene *rfbE*, designed through the use of Primer Designer Software (v1.13; PremierBiosoft) and manufactured by Sigma’s oligonucleotide synthesis service (for LAMP primer information, refer to Table 7). The format of samples tested with LAMP was as according to the manufacturer’s recommendations of 15 µL of Master Mix, 5 µL of Primers, and 5 µL of sample template per reaction. The concentration of the primers in each reaction were 5 pM each of F3 and B3, 10 pM of LoopF and LoopB, and 20 pM of FIP and BIP. The reaction parameters recommended the use of an OptiGene Genie II assay platform for thermocycling conditions, but as this was temporarily unavailable, the reactions were first attempted on the AB Biosystems Viiia7 thermocycler with similar conditions. For the Viiia7, each of the samples to be tested were placed into a 96-well qPCR plate and subjected to the following conditions; 65°C for 60 minutes, followed by an annealing step of 98°C for 2 minutes with temperature ramping of 1.6°C/s. As the master mix contains only SYBR green fluorescent dye, the Viiia7 was set to read SYBR green fluorescence only. The reaction well volume was set at 20 µL per well. On the Genie platform, the master mix, primers and samples were prepared as described above and were loaded into provided custom tubes and placed in slots on the top of the instrument. The assay conditions were 65°C for 30 minutes followed by a single annealing step of 98°C with ramping at 0.1°C per minute. As there were issues with transferring the data from the platform to the included software on the researcher’s computer, screenshots of the relevant results are provided in the results section.
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5′-3′)</th>
<th>Position</th>
<th>Tm (°C)</th>
</tr>
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<tbody>
<tr>
<td>F3</td>
<td>TTCACACTTATTGGATGGTCTC</td>
<td>943</td>
<td>60.1</td>
</tr>
<tr>
<td>B3</td>
<td>TAACTTGCTCATTGATAGGC</td>
<td>1,167</td>
<td>60.2</td>
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<tr>
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<td>TGCAAGGTGATTCCATTAATTCC</td>
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<td>61</td>
</tr>
<tr>
<td>LoopB</td>
<td>AGCACCCCTATAGCTGAGGAT</td>
<td>1,090</td>
<td>62</td>
</tr>
<tr>
<td>F2 (FIP)</td>
<td>ATTCTAACTAGGACCACGAGA</td>
<td>966</td>
<td>60</td>
</tr>
<tr>
<td>F1c (FIP)</td>
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<td>64.9</td>
</tr>
<tr>
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<td>TTAATCCACGCAACCACAA</td>
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<td>B1c (BIP)</td>
<td>TCCACACGATGCAATGTACTC</td>
<td>1,054</td>
<td>65</td>
</tr>
</tbody>
</table>

*rfbE*: perosamine synthetase homolog [*Escherichia coli*, O157:H7 strain 86-24, Genomic, 1287 nt]
3.2.19 ESR serotyping

Samples that had tested positive for stx1 and/or stx2 were sent to the National Centre for Biodefense and Infectious Diseases at ESR, Upper Hutt, for on-site PCR serotyping. The assay in use at ESR is used to identify the wzx gene, which in E. coli encodes the O-antigen flippase within the O-antigen gene cluster. The wzx gene, along with a variety of other serotype-specific genes, show variability between serogroups for use as a diagnostic marker of the O-antigen type, although the combined assay in use at ESR can only deduce if the genes in the samples match the gene cassettes found in O26, O45, O103, O111, O121, or O145 and O157 serotypes only. Samples that were positive for O157 in both the LAMP assay and the wzx assay were also subjected to a PCR assay to determine their H (flagellar) antigen type through variations of the flagellar antigen produced by the flic gene.
3.3 Results

3.3.1 PCR validation assays and amplification efficiency check

Each of the individual probe and primer sets were tested individually against DNA extracted from the lab cultured STEC strains, and were found to amplify sequences as predicted (eae amplified only in O111:K88:H-, stxl amplified only in O91:H- and stx2 only in O130:H11, with uidA amplifying in all E. coli samples). A O84:HNM later sourced from ESR was found to amplify stx2, contrary to reference information, although recurring issues with gel purification prevented sequencing. Similarly, an stx- O157:H-reference strain, to be used as a positive control in LAMP testing, was found to be eae+ (see Table 3). The amplification efficiencies of the individual validation assays of the uidA, eae, stxl, and stx2 probe and primer sets can be viewed in Figures 11 (uidA, eae) and 12 (stxl, stx2). The efficiency graphs are essentially a graphical representation of a titration of STEC DNA (in this case, the DNA from a phenol-chloroform extracted O111:K88:H- culture combined with similarly extracted O130:H11 DNA and O91:H- serially diluted in a ten-fold range of 1 ng/reaction – 0.001 ng/reaction and assayed in triplicate). The efficiency graphs themselves display plotted Ct values in a linear regression for each of the genes at the dilutions of DNA shown. Once the values were plotted, the Applied Biosystems Viia7 software was used to analyse the slope of the curve to determine the amplification efficiency (how effectively the reaction can double the DNA strands per PCR cycle, expressed as a percentage in the ‘Eff96’ category (see Figures 11 and 12)). All of the amplifications were noted to have performed efficiently, with upwards of 97% efficiency as calculated by the Viia7 software. The software also noted a high coefficient of determination (R²) value and low error for each of the standard curves generated, which indicates that there was little variation in triplicate repeats of the assay.

3.3.1.1 PCR amplicon size check

The gel photo of the amplified gel fragments is displayed in Figure 13, alongside an Invitrogen TrackIt 1Kb Plus (Cat no 10488-085) molecular ladder. The sequence fragments of uidA and eae can be viewed in the wells next to the 100bp ladder fragment, which is consistent with the sizes predicted with the Primer Express software. Due to an error in dispensing, the stxl and stx2 fragments were combined in the same gel well, and
Figure 11. Amplification efficiencies of each of the individual assays to be merged in the multiplex reaction: (a) *uidA* probe and primer set; (b) *eae* probe and primer set. Both efficiencies are noted to be in excess of 97%. The efficiencies are an indicator of the ability of the reaction to effectively double the target sequence in each thermal cycle.
Figure 12. Amplification efficiencies of each of the individual assays to be merged in the multiplex reaction: (a) *stx1* probe and primer set; (b) *stx2* probe and primer set. Both efficiencies are noted to be in excess of 97%. The efficiencies are an indicator of the ability of the reaction to effectively double the target sequence in each thermal cycle.
Figure 13. Gel fragments of O84:HNM amplified using the separate assays of the qRT-PCR multiplex (uidA, eae, stx1 and stx2). The predicted fragment sizes for *uidA*, *eae*, *stx1* and *stx2* were 94 bp, 102 bp, 107 bp and 130 bp, respectively. All fragments were of the correct size, running close to the 100 bp ladder fragment. Due to a pipetting error, the *stx1* and *stx2* post-PCR amplified fragments were added to the same well, and so cannot be distinguished due to a similar bp size. The ladder used in this gel is the Invitrogen 1 kb Plus (Cat. No. 10787-018).
so the two cannot be distinguished owing to their similar size, but the gel fragment size is consistent with the expected size for both fragments.

3.3.1.2 *Multiplex amplification efficiency comparison*

The results garnered in the separate amplification efficiency testings were compared with a similar assay incorporating all of the surveyed probes and primer sets in concert within one PCR mix. As with the separate assays, the multiplex assay was completed with a five point ten-fold dilution range (1 ng/reaction – 0.001 ng/reaction) of appropriately extracted O111:K88:H-, O91:H-, and O130:H11 DNA. The AB Biosystems Viia7 platform appeared to be able to discriminate between the similar wavelengths of the fluorescent tags of the *stx1* (560 nm) and *stx2* (555 nm) probes without incident. The amplification efficiencies are recorded in Figure 14 (*uidA, eae*) and 15 (*stx1, stx2*). The amplification of one reaction, *eae*, was noted to have had a decrease in its efficiency (82.772%; down from an efficiency of 98.748% when conducted as a separate assay). However, other tests of this probe and primer set completed after this particular assay were found to have high efficiency ratings (see Figure 16(b)). Examining the amplification plots of the *eae* assay in the multiplex PCR revealed a number of outliers (see Figure 17), and once these were omitted from statistical analyses, the amplification efficiency was noted to increase considerably.

3.3.2 *PCR extraction method yield comparison*

3.3.2.1 *Relative DNA yield by cycle threshold values*

The *uidA* amplification plots of the O111:K88:H- culture as extracted by phenol-chloroform, PrepMan Ultra kit, Chelex-100 resin, and a simple boiling protocol is shown in Figure 18. As the assay functions as a real-time qPCR, the target DNA sequence doubles with every thermal cycle, and the fluorescent probes will fluoresce as they attach to the sequences being amplified. The sample with the greatest yield of *E. coli* DNA recoverable after extraction will reach the threshold fluorescence (a default threshold set by the Viia7 software) first. The sample processed with phenol-chloroform had the highest yield of DNA from the same broth culture relative to the other methods. Surprisingly, Chelex-100 was found to have a greater yield than that of PrepMan, with the boiling method returning the lowest comparative yield. The concentration of the extracted samples as determined by UV spectroscopy are displayed in Appendix 5.
Figure 14. Amplification efficiencies of each of the assays after testing in one combined reaction: (a) *uidA* probe and primer set; (b) *eae*. No significant differences in the amplification efficiencies were noted with the exception of *eae*, which dropped to 82.772% as computed by the Viia7 software. The efficiencies are an indicator of the ability of the reaction to effectively double the target sequence in each thermal cycle. The high efficiency rating of the *uidA* assay in the multiplex (comparable to the efficiency seen in the individual *uidA* reaction in Figure 11) indicates it is unaffected by the other reactions taking place in the multiplex.
Figure 15. Amplification efficiencies of each of the assays after testing in one reaction: (a) stxl (b) stx2. No significant differences in the amplification efficiencies were noted. The high efficiency rating of the stxl and stx2 assays in the multiplex (comparable to the efficiency seen in the individual stxl and stx2 reaction in Figure 12) indicates they are unaffected by the other reactions taking place in the multiplex. A graphical error occurred with the Viia7 software when generating the curve of stx2, and a misaligned curve can be seen despite an $R^2$ value of 0.955.
Figure 16. Investigating variation seen in the eae amplification efficiency in multiple testing. Figure 16(a) depicts the eae standard curve completed as part of a multiplex reaction as was shown in Figure 14(b). Blue arrows indicate values amongst the triplicate repeats that appear to be outliers. A similar assay (shown as Figure 16(b)) of a standard comprised of O84:HNM completed near the time of this assay and with the same reaction conditions was noted to have markedly better amplification efficiency by the Viia7 despite a considerable outlier. Adjustments to correct the efficiency by eliminating outlying values are depicted in Figure 17.
Figure 17. Investigating variation seen in the *eae* amplification efficiency in multiple testing. Upon examining the corresponding quantities (0.1 ng and 0.0001 ng) on the amplification plot of the *eae* assay (shown in Figure 16(a)), the outlier values were again apparent (red arrows). Once these values were removed from statistical analyses, the corrected *eae* standard curve showed an improved efficiency rating as seen in Figure 17(b) (89.483%).
Figure 18. Relative DNA yield comparison of a broth culture. A single 6 hour TSB culture of *E. coli* O111:K88:H11 was extracted by four DNA extraction methods; phenol-chloroform phase separation with salt precipitation, PrepMan Ultra (a kit-based protocol), boiling with Chelex-100 resin, or by a simple boiling protocol. The extracted DNA samples were then run under identical PCR conditions on an AB Biotec Systems qPCR Viia7 thermocycler, amplifying a sequence within the *E. coli* uidA gene. The method with the highest comparative yield of DNA is that of the phenol-chloroform extraction, which has the lowest Ct value. The Chelex-100 resin is noted to have a DNA yield comparable to that of the PrepMan method.
3.3.2.2 Gel photos of extracted DNA

Photos of the gels used to examine the physical state of the extracted DNAs are displayed in Figure 19. The phenol-chloroform extracted culture of O91:H- showed the most prominent DNA streak when run on an electrophoretic gel, followed by the Chelex-100, then boiling, with PrepMan having the least substantive streak. The positions and size of the streaks on the gel provide information on the degree of degradation that the DNA encountered during the respective extraction procedures, as larger, undamaged DNA fragments migrate more slowly across the gel. The DNA processed by the boiling method can be seen as a long faint streak running near the bottom of the gel, indicating that the DNA itself has fragmented into strands small enough to run ahead of the 2,000 fragment seen in the molecular ladder. The phenol-chloroform extracted DNA can be seen to have fragments running near the 2,000 bp ladder mark on its respective gel, suggesting some fragmentation, but most of the DNA can be seen in a band well above the 12,000 bp mark, indicating that most of the recovered DNA is considerably less fragmented. The intensity of ethidium bromide staining also suggests the phenol-chloroform bands contained the highest concentration of DNA. The Chelex-100 band, while not as intense as the phenol-chloroform extracted material, remained in a relatively tight streak near the 40,000 bp fragment of the DNA ladder. The PrepMan streak is barely visible in the circled region of the gel in Figure 19(b), running ahead of the 2,000 bp ladder fragment, although not as dispersed as that of the material in the boiling lane.

3.3.3 PCR-based quantitation

In order to arrive at a representative weight per genome of stx-bearing E. coli, all of the completed chromosomal E. coli FASTA sequences were downloaded from the NCBI database and searched for probe sequences using pDRAW software. An example of a pDRAW sequence search is demonstrated in Figure 20. The genome sizes of the strains (n = 17) that were found to carry one or more of the stx genes (displayed in Appendix 6) were then graphed using PRISM software alongside a sample (Figure 21) of non-stx carrying E. coli (n = 29; see Appendix 7), and together with the mean GC percentage, the mean genome size was used as the basis of calculations to determine a representative weight per stx-bearing genome (calculations expressed in Appendix 8). For stx-bearing E. coli, this number was determined to be 1.7 x 10⁵ genomes per ng of DNA. With this information, and using an appropriate standard curve, it was possible to programme the
Figure 19. Gel photos of 6 hour TSB cultures of *E. coli* O91:H- DNA extracted by either phenol-chloroform, Chelex-100, PrepMan or boiling methods. Figure 19(a) depicts the culture processed by boiling and PrepMan extraction methods run against a 1Kb extension ladder (Invitrogen™). The DNA processed by boiling can be seen in a long streak towards the bottom of its lane, while the DNA processed by Chelex (arrow) remains near the loading wells, roughly equivalent to the 40,000 Kb ladder marker. The DNA processed by Phenol-Chloroform and PrepMan can be seen in Figure 19(b); Phenol has the most easily visible DNA streak running almost the length of the gel, whereas the PrepMan streak is barely visible (circled) at about 2,000 Kb as compared to the Invitrogen 1Kb Plus Ladder (Cat. No. 10787-018) used in this gel. The negative controls shown in both gels were sterile TSB processed along with the live cultures.
Figure 20. Example of a genome sequence search conducted using pDRAW (ACACLONE) software on *E. coli* FASTA sequences sourced from the NCBI *E.coli* ‘Genome Assembly and Annotation Report’ webpage. Several genomes containing *stx* genes were used to calculate a representative STEC mean genome size.
Figure 21. Box and whiskers plot of the genome sizes of NCBI-sourced FASTA sequences with and without stx genes (those displayed in Tables 5 and 6). The means (depicted by a cross symbol) of the stx\(^+\) (5.609 Mb) and stx\(^-\) (4.984 Mb) groups were found to be significantly different (p = <0.0001). The mean of the stx-bearing E. coli (5.609 Mb) was deemed a suitable basis of molecular weight calculations for stx\(^+\) E. coli based on the apparent absence of outliers and on the low coefficient of variation (2.26%) seen amongst the genome sizes of stx-bearing E. coli.
Viia7 software to provide an estimate of the number of stx-bearing genomes within a given well, whether this be DNA extracted from *E. coli* broth culture or faecal extracts.

### 3.3.4 Stationary-phase O84:HNM growth curve

The OD\textsubscript{600nm} readings from the eighteen hour O84:HNM culture experiment can be viewed graphically in Figure 22, along with the corresponding CFU (as calculated based on the O84:HNM OD\textsubscript{600nm} curve in Figure 7). The growth curve results showed that viable stationary phase *E. coli* O84:HNM (for use in later spiking experiments) could be collected from overnight cultures before the culture entered death phase. The culture that was mixed and measured only every three hours appeared to have markedly lower OD\textsubscript{600nm} reads from 6 hours onwards, and shows the effect of agitation on *E. coli* broth culture growth.

### 3.3.5 Preliminary Faecal screening

Five faecal samples (designated α, β, γ, θ, and μ) were sourced from SCL and screened for *eae, stx1*, and *stx2* by testing with the multiplex after a 3 hour TSB pre-enrichment to select a sample for faecal spiking experimentation that did not contain endogenous *stx*-carrying bacteria. Sample α was found to be positive for *stx1* and *stx2* and the others tested negative for all but *uidA*. Sample β had a large number of *E. coli* genomes as indicated its mean *uidA* Ct (15. 924) and sample γ had a lesser amount (mean Ct value of 25.61). Sample β and γ were chosen for subsequent faecal spiking so that comparisons in the Ct values between the two could be used to determine if the amount of commensal *E. coli* had any effect on the sensitivity of the test.

### 3.3.6 Multiplex sensitivity testing by faecal spiking

The common limit of detection of the multiplex for the pathogenic genes *stx1*, *stx2*, and *eae* within a faecal matrix was deemed to be 2 x 10\textsuperscript{3} CFU (of *E. coli* O84:HNM) per mL of well mixed, unincubated faecal-broth culture (1 part faeces to 9 parts culture medium) or 2 x10\textsuperscript{4} CFU per g of faeces for faecal samples β and γ. This was determined by examining the fluorescent signals of the corresponding probe markers (Cal Fluor 560 for *stx1* and TAMRA 555 for *stx2*) to determine at which spiking concentration the signals could no longer be reliably detected (at least two of the three replicate tests at a certain spike concentration had to provide a change in fluorescence discernible with AB Biosystems 7500 software, as displayed in Figure 23). The limit of detection for the *eae*
Figure 22. Calculated (log-transformed) CFU mL$^{-1}$ of the 18 hour TSB cultures of O84:HNM E. coli. The OD/CFU measurements indicate that the rate of growth amongst O84:HNM E. coli slows after 6 hours when mixed and measured on a 3-hourly basis, reaching a mean (non-log transformed) OD of 0.419, as opposed to a mean OD of 0.723 when mixed and measured hourly. The data indicates that overnight cultures (c. 18 hours) can be used to acquire stationary-phase E. coli before such cultures enter death phase (which would be normally be indicated by a plateau of OD readings). This is done to ensure that the O84:HNM OD curve (depicted in Figure 7) can be used to infer CFU data for overnight cultures without discrepancies in CFU and OD caused by dying bacteria.
Figure 23. Limit of sensitivity as determined by faecal spike assay. Faecal samples β and γ were spiked with known CFU per g of *E. coli* O84:HNM and extracted with Chelex in conditions matching that intended for the samples in the greater prevalence study (these spiked samples were not incubated). The limit of sensitivity was defined as the lowest concentration that all of the pathogenic genes (*eae, stx1*, and *stx2*) could be detected (at least two of the triplicate tests giving a discernible increase in fluorescence). While *eae* could be detected in the faecal samples inoculated with $2 \times 10^3$ CFU of bacteria per g faeces (Figure 23(b)), the lowest common limit was found to be $2 \times 10^4$ CFU per g of faeces (as depicted in faecal sample β in Figure 23(a); the same result was garnered from sample γ).
gene was lower than that of stxl and stx2, with noticeable amplification of eae+ in samples spiked with 2 x 10^3 O84:HNM CFU per g of faeces. After 3 hours of incubation, all three pathogenic genes could be detected in both faecal cultures that had been spiked with 2 x 10^2 CFU O84:HNM per g of faeces, indicating the spike bacteria were able to be detected at the multiplex’s limit of detection established by the ‘0h’ cultures.

The similarly designed experiment described in the methods section featuring exponential-phase O84:HNM used to spike the β and γ faecal cultures without incubation was found to have the same limit of detection (2 x 10^3 O84:HNM CFU per mL of well mixed, unincubated faecal-broth culture or 2 x10^4 CFU per g of faeces).

3.3.7 Enumeration of spike replicate

The overnight TSA plate culture counts of the recreated spikes (see section 3.2.11) plated before incubation were counted and the corresponding CFU mL⁻¹ of TSB were calculated. The plate counts of the broth inoculated with 2 x 10^5 CFU equated to 2.2 x 10^5 CFU in the whole 10 mL broth. Similarly, the broth inoculated with 2 x 10^4 CFU spike equated to 2.2 x 10^4 CFU in total in the 10 mL broth. The broth inoculated with 2 x 10^3 equated to 2,260 CFU in 10 mL of broth. Although this test could have benefited from repeat plating of these cultures, these results are consistent with the O84:HNM OD_{600nm} standard curve being reasonably accurate, and that the values expressed in faecal spiking experimentation were not influenced by a failure in the O84:HNM OD_{600nm} to estimate bacterial CFU concentrations. The plate cultures derived from the same TSB cultures that had undergone 3 hours of incubation showed 213 colonies for the culture inoculated with 2 x 10^2 O84:HNM, which equated to 21,300 CFU in 10 mL of broth. The post-incubation culture inoculated with 2 x 10^1 O84:HNM was found to have 3,800 CFU in 10 mL of broth. As this experiment was in part completed to determine if O84:HNM had a significantly faster growth rate than other E. coli, the equivalent CFU mL⁻¹ (of broth) values were plotted against theoretical optimal E. coli growth conditions (see Figures 24 and 25). The theoretical optimal conditions themselves were plotted by doubling the inoculum concentration for 9 growth cycles of 20 minutes. The values were found to be closest to a model assuming that the E. coli did not begin doubling for two typical E. coli doubling cycles (40 minutes) once incubation had begun. These results rule out the possibility of E. coli O84:HNM having a markedly reduced lag phase.
Figure 24. Growth characteristics of stationary-phase O84:HNM. Stationary-phase O84:HNM were used to inoculate TSB at the concentration shown to elucidate the growth characteristics of this strain in the absence of faeces. The plate count of the culture (yellow dot) retrieved after 3 hours of incubation at 37°C is consistent with the model proposing that the bacteria spend two growth cycles (40 minutes) in lag phase.
Figure 25. Growth characteristics of stationary-phase O84:HNM. Stationary-phase O84:HNM were used to inoculate TSB at the concentration shown to elucidate the growth characteristics of this strain in the absence of faeces. The plate count of the culture retrieved after 3 hours of incubation at 37°C (yellow dot) is consistent with the model proposing that the bacteria spend two growth cycles (40 minutes) in lag phase.
3.3.8 Plate counts, NanoDrop readings and calculated Ct values comparison

The plate counts, NanoDrop readings and genome estimates based on Ct readings were compared to examine potential discrepancies. The three *E. coli* O130:H11 cultures returned plate counts that equated to $1.88 \times 10^9$, $2.03 \times 10^9$ and $2.14 \times 10^9$. The same cultures, when quantitated by averaging triplicate NanoDrop reads of the extracted DNA and multiplying this value by the total volume of the extracted DNA, returned values of $7.77 \times 10^9$, $6.92 \times 10^9$, and $8.43 \times 10^9$ genomes per mL of phenol-chloroform extracted broth. When 1 µL of these extracted DNA samples were assayed alongside a (phenol-chloroform extracted) O130 standard curve, the values of the samples were determined to be $6.85 \times 10^9$, $5.58 \times 10^9$ and $7.59 \times 10^9$ genomes per mL of phenol-chloroform extracted broth.

3.3.9 ROX-inclusive master mix validation

Amongst the four assays within the multiplex, only the *uidA* assay showed any amplification when used with TaqMan Universal Master Mix, and so the endogenous dye therein could not be used to correct for the algorithmic correction problem encountered with some *eae* assays. As *eae*+ samples could be discriminated from anomalous readings with use of the ‘multicomponent plot’ function of the Viia7 software (as shown in Figures 26 and 27), it was decided to proceed with the use of ToughMix for the main faecal assays.

3.3.10 384-well plate validation assay

Prior to the 384-well validation, it was found that the Viia7 software required a recalibration for the detection of Quasar 670, and it was recommended that 300 nM of *eae* probe per reaction be used for assays involving 384 well plates due to an inability of the instrument to detect Quasar at the optimised concentration of 80 or 120 nM. The initial assay of a manually loaded 384 well plate was otherwise completed without incident; there was no noticeable difference in fluorescence generated or in the sensitivity of the multiplex to detect the phenol-chloroform extracted DNA standard compared to assays of a 20 µL volume on 96 well plates. An initial test involving the loading of the DNA standard and selected processed faecal samples was completed to satisfaction. The first 32 Chelex-processed faecal samples collected for the prevalence study were assayed
Figure 26. Anomalous Quasar 670 correction problem. An example of a recurring algorithmic correction problem. While an attempt to use a qPCR mix with an endogenous dye for signal normalisation was unsuccessful, it was found that truly positive samples could be discriminated by examination of the signal change under the ‘Multicomponent plot’ feature of Viia7 and 7500 on a case-by-case basis.
Figure 27. Multicomponent plots. While an attempt to use a qPCR mix with an endogenous dye for signal normalisation of anomalous Quasar 670 fluorescence signals was unsuccessful, it was found that truly positive samples could be discriminated by examination of the Quasar signal change under the ‘Multicomponent plot’ feature of Viia7 and 7500 on a case-by-case basis. Graph (a) depicts negative samples that showed the anomalous pattern on the amplification chart, and graph (b) depicts a truly positive sample.
with the multiplex as part of the 384-well plate validations, the results of which are included in the ‘results of main assays’ subsection of the ‘main faecal assays’ section.

3.3.11 Main faecal assays

3.3.11.1 Robot-Loaded 384 plate assays
The initial test of function using 384-well qPCR plates performed as expected; both the O84:HNM DNA standard and the select faecal specimens produced clear signals with no clear indication that the robot had failed to load the specimens. The subsequent loading of the processed faecal samples into the 384-well plate proceeded without incident, however the amplification failed, with the wells appearing under filled. A cursory test of robotic dispensation of a viscous solution with a water and glycerol solution indicated the robot loader was dispensing appropriately. A second attempt of the robot loading was met with mixed results; despite adapting the loading protocol to account for possible dispensing inefficiencies, much of the right hand side of the 384-well plate appeared underfilled, and many of the samples that appeared filled showed no amplification of the tentative faecal sample amplification control $uidA$. It was decided to assay the remaining faecal samples (and those failed samples from the reattempted 384-well assay) on 96-well plates with manual loading and a reduced volume.

3.3.11.2 Validation of reduced volume 96-well assay
The validation of the multiplex with conditions of reduced volume performed as expected; the assay could detect 100 ng of O84:HNM without an appreciable loss of florescence when the reagent volumes (except the sample) were reduced by factor of two from 20 µL per well to 10 µL per well.

3.3.11.3 Remainder of main assay testing
The remainder of processed faecal samples were processed on 96-well well assay plates on the AB Biosystems 7500 thermocycler without need for assay redesign, although some samples did not display any amplification.

3.3.11.4 Results of main assays
Collectively, of the 522 processed diarrhoeic stool samples that were tested across the assay types described above, 8 were recorded as testing positive for either $stx1$ or $stx2$, equating to a prevalence of 1.53% (0.66 – 2.99%; 95% CI). The patient details of the $stx$
positive samples are recorded in Table 8; this table incorporates serotype data from the ESR testing section and samples that were in the *H. pylori* group. Amongst the 50 samples that had been set aside for *H. pylori* testing, 3 tested positive for *stx*1 or *stx*2, equating to a prevalence of 6% (1.25 – 16.54%; 95% CI) in this group (these samples tested negative for *H. pylori*). A Fisher’s exact test could not determine a statistically significant difference in the STEC prevalences between these two groups (*p* = 0.063; 95% CI). No STEC were found within the 26 faecal samples set aside for faecal calprotectin testing. Overall, 26 samples tested positive for *eae*. Amongst the main pool of samples, 23 (4.41%) samples tested positive for *eae* (2.96 – 6.53%; 95% CI), two of which were later found to be serotype O157:H- (for a breakdown of these samples and related information, refer to Table 9). Two specimens amongst the faecal calprotectin samples (n = 26) were found to be positive for *eae*, but the prevalence of *eae*+ only *E. coli* in this group was not found to be significantly different from that of the main group (*p* = 0.298827; 95% CI).

### 3.3.12 PVPP column testing

#### Initial test of PVPP processing of faecal samples and BSA assay

The initial test of PVPP columns and BSA, displayed as a comparison of the triplicate Ct values of the specimens with and without the post-extraction treatments, are displayed in Figure 28. The four Chelex-processed samples chosen were #157, a specimen that had displayed an acceptable *uidA* amplification on the reattempted 384-well assay, and samples #131, #134, and #139, which showed no amplification on the 384-well assay (although it remained unclear at this time whether the lack of *uidA* amplification in these wells was as a result of PCR inhibition or the robotic platform not having dispensed the processed faecal samples appropriately). The Ct values of #139 were the most consistent with the expected pattern of inhibition; the assay failed to amplify but was able to with PVPP processing. The variability of the triplicates of sample #131 appeared to decrease markedly with PVPP processing. Sample #134 did amplify in this testing without PVPP, suggesting the lack of *uidA* amplification seen in this well on the 384-well assay prior was more likely as a result of mechanical error by the platform. The Cts of sample #157 and #134 actually increased with PVPP processing. The addition of BSA to qPCR mixes assaying the PVPP-processed samples resulted in no significant change in Ct values. The test of the 96-well Silicon-A-HRC purification plate with sample #139 indicated that the plate could not improve Cts as demonstrated by tests with the individual microcentrifuge
TABLE 8: Patient status, serotype and \textit{stx} types for \textit{stx}+ faecal samples detected

<table>
<thead>
<tr>
<th>Sample number designate</th>
<th>Age</th>
<th>In/Out patient</th>
<th>Serotype (ESR)</th>
<th>\textit{eae} Status</th>
<th>\textit{Stx1} status</th>
<th>\textit{Stx2} status</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>≥12</td>
<td>Inpatient</td>
<td>?</td>
<td></td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>≥12</td>
<td>Outpatient</td>
<td>?</td>
<td></td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>≥12</td>
<td>Outpatient</td>
<td>?</td>
<td></td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>271</td>
<td>≥12</td>
<td>Outpatient</td>
<td>?</td>
<td></td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>284</td>
<td>≥12</td>
<td>Outpatient</td>
<td>O157+, O103+</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>370</td>
<td>≥12</td>
<td>Outpatient</td>
<td>?</td>
<td></td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>511</td>
<td>≥12</td>
<td>Inpatient</td>
<td>?</td>
<td></td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>547</td>
<td>≥12</td>
<td>Outpatient</td>
<td>O157+, O103?</td>
<td>positive</td>
<td>weakly positive</td>
<td>weakly positive</td>
</tr>
</tbody>
</table>

\textit{Stx}+ samples from the \textit{H. pylori} pool

<table>
<thead>
<tr>
<th>Sample number designate</th>
<th>Age</th>
<th>In/Out patient</th>
<th>Serotype</th>
<th>\textit{eae} Status</th>
<th>\textit{Stx1} status</th>
<th>\textit{Stx2} status</th>
</tr>
</thead>
<tbody>
<tr>
<td>92</td>
<td>≥12</td>
<td>Outpatient</td>
<td>?</td>
<td></td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>456</td>
<td>≥12</td>
<td>Outpatient</td>
<td>?</td>
<td></td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>461</td>
<td>≥12</td>
<td>Outpatient</td>
<td>?</td>
<td></td>
<td>positive</td>
<td>positive</td>
</tr>
</tbody>
</table>
TABLE 9. Patient status, stx types, and other test details for eae+ faecal samples detected  
(continued on next page)

<table>
<thead>
<tr>
<th>Sample number designate</th>
<th>Age</th>
<th>In/Out patient</th>
<th>Stx1 status</th>
<th>Stx2 status</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>≥12</td>
<td>Inpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>&lt;12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>&lt;12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td>NSF colony sent to ESR, no Shiga toxin detected</td>
</tr>
<tr>
<td>89</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>117</td>
<td>≥12</td>
<td>Inpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>≥12</td>
<td>Inpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>266</td>
<td>&lt;12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td>NSF colony sent to ESR, no Shiga toxin detected</td>
</tr>
<tr>
<td>278</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td>NSF colony sent to ESR, no Shiga toxin detected</td>
</tr>
<tr>
<td>284</td>
<td>≥12</td>
<td>Outpatient</td>
<td>positive</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>287</td>
<td>&lt;12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>288</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>291</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td>NSF colony sent to ESR, no Shiga toxin detected</td>
</tr>
<tr>
<td>324</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>344</td>
<td>&lt;12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>378</td>
<td>&lt;12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td>Faecal calprotectin sample; quantitative test negative</td>
</tr>
<tr>
<td>380</td>
<td>&lt;12</td>
<td>Inpatient</td>
<td></td>
<td></td>
<td>Sample tested positive for Campylobacter, Salmonella</td>
</tr>
<tr>
<td>418</td>
<td>≥12</td>
<td>Inpatient</td>
<td></td>
<td></td>
<td>Faecal calprotectin sample; quantitative test negative</td>
</tr>
<tr>
<td>440</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>456</td>
<td>≥12</td>
<td>Outpatient</td>
<td>positive</td>
<td>positive</td>
<td>H. pylori sample (antigen negative)</td>
</tr>
<tr>
<td>489</td>
<td>&lt;12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>506</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>524</td>
<td>≥12</td>
<td>Inpatient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample number designate</td>
<td>Age</td>
<td>In/Out patient</td>
<td>Stx1 status</td>
<td>Stx2 status</td>
<td>Details</td>
</tr>
<tr>
<td>-------------------------</td>
<td>------</td>
<td>----------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>547</td>
<td>≥12</td>
<td>Outpatient</td>
<td>weakly positive</td>
<td>weakly positive</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>≥12</td>
<td>Outpatient</td>
<td></td>
<td></td>
<td>NSF = Sorbitol non-fermenting.</td>
</tr>
</tbody>
</table>
Figure 28. Test of function with PVPP columns. Four Chelex extracted faecal samples of dubious assay status were processed with PVPP columns and part of these processed samples were also amplified in a qPCR mix incorporating BSA to determine if PVPP or BSA made any discernible improvement in Ct or permitted amplification to occur. The red lines indicate the statistical significance between groups of Ct values by concentration, as determined by ANOVA analysis (GraphPad Prism software); * denotes $p < 0.05$, ** denotes $p < 0.05$, and *** denotes $p < 0.01$. The PVPP processing did appear to permit sample #139 to amplify and appeared to reduce the variability in Ct values seen in #131, although it did induce significant increases in the Ct values of #134 and #157. The only significant effect on Ct values induced by adding BSA to the qPCR mix appears to be as a result of a considerable (disregarded) outlier in sample #139. As the PVPP columns could demonstrably remove putatively inhibitory substances from samples with poor amplification patterns, it was decided to process the remainder of such samples to see if the amplification of other samples could be improved.
PVPP columns. Based on these results, it was decided that it would be worthwhile to process the samples that had shown poor amplification of \textit{uidA} in the main assays with the individual PVPP columns, to see if any improvement in Ct values could be made.

### 3.3.12.2 Main PVPP processing and retesting of questionable samples

Of the 95 samples from the main assays retested after PVPP column processing, only five appeared to have any noticeable decrease in Ct values. Most samples showed little or no improvement with PVPP processing, with 26 showing no amplification before or after PVPP processing. Four samples that had high but very close duplicate Ct values in the main assays showed complete degradation of their signals following PVPP processing.

### 3.3.12.3 Internal amplification testing of selected PVPP-processed samples

The Ct values of the internal amplification control are displayed in Figure 29. ANOVA analysis showed no significant difference between Ct values of the internal amplification control in wells that contained any of the processed faecal samples and that of the included negative (no added DNA) control. Similarly, no significant difference in the internal amplification control Ct values could be determined when comparing each faecal specimen that had been processed by PVPP with the equivalent specimen that had not undergone PVPP processing.

### 3.3.13 LAMP based detection of O157 serotypes

The initial test of the LAMP primers and PCR mix on the Viia7 thermocycler produced no amplification in any of the \textit{stx}+ samples or controls, despite replication of the cycling parameters described in the PCR mix insert. When the assays were completed on the Genie platform, faecal samples #284 and #547 showed SYBR green fluorescence comparable to that of a Chelex-extracted O157+ control (the #547 and positive control are depicted in Figure 30; Figures 30 and 31 are provided as photographs of the Genie II display due to a malfunction of the platform to transfer data via a USB port). Melt curves provided by the software (occurring during the annealing stage of the programmed parameters) showed the products in the faecal samples were in consensus with the provided control (see Figure 31).
Figure 29. Internal amplification check of resolutely negative extracted samples. An internal amplification probe (pChook IAC) was added to a qPCR mix and used to determine whether a subsample of Chelex-extracted specimens that showed no amplification of target sequences were under the influence of PCR inhibitors. The assay also incorporated a test of PVPP processing; each sample’s internal amplification Ct values would be compared against the same specimen that had undergone PVPP processing as well as against a negative control (to which no DNA had been added, shown in red on this graph). None of the Ct values showed a significant difference from the ‘No DNA’ control when analysed by ANOVA analysis (95% CI), signifying that samples were not under the influence of PCR inhibitors.
Figure 30. Genie II display featuring O157+ sample amplification. Faecal sample #284 (green line) is shown amplifying alongside a positive control (Chelex-100 culture of O157+ E. coli). A melting curve analysis of these samples can be viewed in Figure 31.
Figure 31. Genie II display featuring O157+ melt curve analysis. Faecal sample #284 (green line) can be seen here with a melting signature equivalent to that of an *E. coli* O157+ control. An accompanying table display listed the sample and control to have peak annealing temperatures of 84.65°C and 84.64°C, respectively. A very similar temperature (84.77°C) was recorded for the other faecal sample designated O157+, sample #547.
3.3.14 ESR serotyping

The serotyping results from ESR indicated that faecal specimens #284 and #547 were positive for O157, in concordance with the LAMP-based serotyping carried out by the researcher. #284 also tested positive for O130. A trace of O130 was also detected in #547, however the low intensity of the fluorescent signal meant this result was not conclusive. Both #284 and #547 tested negative for the common O157 flagellar antigen H7. None of the stx\(^+\) samples other than #284 or #547 could be serotyped, signifying that none of the surveyed stx\(^+\) E. coli were of the serotypes O26, O45, O103, O111, O121, or O145.
3.4 Discussion

3.4.1 PCR validation assays and amplification efficiency checks

The amplification efficiencies were found to be over 97% when tested separately. Ideally, qPCR efficiencies should be 90-105% \( \text{\textsuperscript{132}} \), although 100% efficiency as computed from a five order-of-magnitude dilution series is recommended for the quantification of \textit{E. coli} from environmental samples \( \text{\textsuperscript{93}} \). The primer sequences used in this project were adapted from sequences developed for multiplex use by Nielsen \textit{et al}, and have been previously tested against GenBank sequences to ensure specificity \( \text{\textsuperscript{46}} \). A cursory test of specificity was carried out through individual \textit{eaeA}, \textit{stx1}, \textit{stx2} amplification reactions with strains known to be lacking each of these genes. Multiplexing PCR reactions can elicit the formation of dimerised or secondary structures that can selectively impede amplification of one or more targets \( \text{\textsuperscript{111}, 133} \). The formation of such structures cannot be reliably predicted by primer design software, and so empirical testing is usually necessary to determine if any one primer pair interferes with other amplifications in the multiplex \( \text{\textsuperscript{134}} \). The forward and reverse primer sequences for \textit{uidA} were redesigned for this project to have a closer annealing temperature to that of the other primers in the multiplex (as an additional measure to improve amplification consistency \( \text{\textsuperscript{133}} \)). The multiplex efficiency test was necessary to ensure that the new primers did not impinge upon the efficiency of the other reactions. It was found that the \textit{eae} reaction efficiency was adversely affected by multiplexing. However, on closer examination, it was found that the poor reaction efficiency was as a result of variation between triplicate wells probably due to technical error and this did not occur in subsequent experiments. The gel electrophoresis of the separate assays showed that fragments of the correct size were being amplified, and although the \textit{stx2} fragment could not be differentiated from \textit{stx1} on the gel, subsequent RT-PCR assays showed amplification of the target in expected \textit{E. coli} strains. In review, the fragment gel electrophoresis should have been repeated to clearly distinguish \textit{stx1} and \textit{stx2} fragments and mitigate the dark banding caused by the loading dye. As the primers had undergone specificity testing in two papers \( \text{\textsuperscript{46, 82}} \), and as the gel was not inconsistent with the expected fragment sizes, a repeat was postponed at the time in favour of other experiments owing to time constraints. Several attempts to sequence fragments from this and a similar gel with the use of a Wizard\textsuperscript{\textregistered} SV Gel and PCR Clean-Up System failed.
3.4.2 PCR extraction method yield comparison

The comparison of the four extraction methods by RT-PCR and gel electrophoresis revealed that the phenol-chloroform extraction gave the greatest yield of DNA from a pure broth culture compared to the other extraction methods. Chelex-100 had the next highest yield, performing better than PrepMan Ultra or the simple boiling protocol. The phenol-chloroform method is regarded as a classic DNA extraction gold standard and is often used as a basis of comparison for the efficacy of DNA extraction methods (128, 130, 135, 136). Surprisingly, Chelex-100 permitted the amplification of a better yield of DNA than the kit-based PrepMan method, and appeared to result in less DNA fragmentation upon DNA analysis. Little information is available on the mode of action of the PrepMan Ultra reagent, but the extraction method has been used in several studies involving the extraction of STEC from pure broth culture, bovine faeces and beef samples (82, 137, 138). PrepMan is not recognised to be the most ideal extraction method, with one study finding a modified TE heat lysis extraction more effective (138), and another correlating the use of larger volumes of the reagent with PCR inhibition (139). Chelex-100 resin is composed of styrene divinylbenzene copolymers containing paired iminodiacetate ions which chelate polyvalent metal ions in solution (140). Chelex-100 is effective over all biological pHs (140), and has been demonstrated to have a extraction efficacy comparable to that of phenol-chloroform in a variety of human tissues (128, 130, 141, 142). Due to concerns of the influence of proteins and other particles left over from Chelex-based extraction on spectroscopic readings, it was decided to measure the effective yield of amplifiable DNA by PCR rather than direct DNA spectroscopy (131). Chelex is speculated to prevent DNA degradation by chelating metal ions that act as catalysts at high temperatures in solutions of low ionic strength (141, 143), while also binding porphyrin inhibitors from blood (126). The boiling method offers nothing in advance of the PrepMan or Chelex methods, relying on temperature and centrifugation of the sample to selectively denature and remove PCR inhibitors.

Cost, sensitivity and practicality of the extraction methods were considered before choosing an ideal method for the project. The phenol-chloroform method, whilst an industry benchmark, was time-consuming to perform on multiple samples, and involved the use of toxic organic solvents not suited to extensive or high throughput testing. The phenol-chloroform protocol also had more washing and transfer steps that could increase the risk of introducing contaminants to the samples (126). The Chelex and PrepMan
methods could be typically completed within one hour without the use of a fume hood, and had demonstrably less DNA fragmentation than the simple boiling extraction (visible as a low molecular sized streak on the gel in Figure 19). The PrepMan Ultra kit was valued at NZ $173.00 for 200 preparations (87¢ per test), while the Chelex-100 resin was valued at NZ $456 for 5500 preparations (8¢ per test). It was decided to proceed with use of Chelex on the basis of cost and the comparative DNA yield as a means to maximise sensitivity. In retrospect, a comparative test of Chelex-100 and PrepMan efficacy in extracting STEC from pre-spiked faecal samples would have been an appropriate accompanying test. An analysis of the four extraction methods would also benefit from an analysis of Ct values versus concentration to control for inconsistencies in amplification efficiency wrought by any particles left over from each extraction method. Ideally, more extensive testing, perhaps comparing more specialised kit-based tests such QIAamp DNA Stool Mini Kit or ZR Fecal DNA MiniPrep to Chelex-100, could be undertaken to give a more thorough assessment of comparative DNA yield and cost effectiveness (136).

3.4.3 PCR-based quantitation

One of the early objectives of the project was to investigate the potential to derive quantitative information on STEC in active infections. The survey of genome sizes of \( stx^+ \) \( E. coli \) provided a representative average genome size (5.609 Mb) from which the number of STEC genomes in a sample (by weight of DNA, 174,000 genomes per ng) could be estimated. Due to differences in chromosomal sizes between STEC and non-pathogenic \( E. coli \) alluded to within the literature (16, 144, 145), size sampling was used to determine a representative average of STEC genome size. It was reasoned that RT-PCR could be used to arrive at estimates of the number of STEC per gram of faeces in a manner similar to other PCR-based studies (82, 93, 146-149), but both the implementation and relevance of quantitation of STEC has proven to be problematic.

While the Viia7 platform could be configured to arrive at estimates of genomes per reaction, discrepancies emerged between the values obtained by plate counts, the values determined by UV spectroscopy of bacterial DNA and the genomes/mL as calculated from Ct values in a subsequent qPCR reaction. These discrepancies were not unexpected, as differences between plate counts and PCR-based techniques can arise through a confluence of the presence of viable but non-culture cells, the aggregation of cells on
plate media and the influence of growth dynamics on genomes per cell (150, 151). These discrepancies could be corrected by deducing the conversion factor for genomes detected by PCR to the number of cells present (150), but further testing was not pursued in favour of other experiments. In retrospect, Chelex-processed faecal samples pre-spiked with known concentrations of STEC on a log_{10} scale, like that performed by Ibekwe et al (93), would have been a more appropriate standard curve for faecal STEC enumeration. The method also needed further testing with a range of faecal samples to determine what proportion of total faecal DNA was lost during Chelex-based extraction before reliable genomes/gram faeces estimates could be placed. Attempts to use the phenol-chloroform extracted pure culture standard curve of O84:HNM to quantify O84:HNM within spiked Chelex-extracted faecal samples yielded estimates inconsistent with the known spike concentration, even when accounting for the different sample extraction volumes generated by the two extraction methods. During the collection phase of this project, a rough estimate of volume was noted for faecal samples that were either too viscous to be pipetted accurately or less than 1 mL. In review, these estimates would have led to further error in genomes/gram estimates had quantitative information been sought. A more appropriate technique would have been to record the net weight of faeces added to the broth universals and to use these values as a basis of subsequent calculations. Growth dynamics of the resident STEC were also considered to be a possible confounder; although the 3 hour TSB pre-enrichment would limit the amount of growth variance between strains, some system of correcting differential growth would have to be implemented. It is difficult to estimate the effects of these enhancements in the method without further testing, and a lack of standardised protocols for the quantification of pathogens in faeces by PCR has not helped (117).

In addition to technical impediments, the nature of STEC infection ultimately limits the relevance of faecal genome estimates in diagnostic practice. The secretion of free Shiga toxin within the mammalian intestine is thought to be mediated primarily by the rare but spontaneous induction of the resident Shiga prophage, such that a subpopulation of the infecting STEC strain lyses and releases toxin and lysogenic phage particles (26). The basal level of this form of expression is recognised to vary from strain to strain (106, 152, 153), and as there are over 150 serotypes of non-O157 STEC associated with human disease (3, 8), it can be seen that the number of resident STEC may not be a reliable indicator of disease severity (59). On the basis of these findings, it was deemed that
quantitative information on STEC infection was not a priority to the project, and subsequent experiments were directed towards maximising sensitivity and adapting the assays to high throughput specifications.

3.4.4 Stationary-phase cultures, faecal spiking

The stationary-phase O84:HNM experiments were conducted to ensure that viable O84:HNM could be obtained from static overnight TSB cultures so as to provide a growth model representative of naturally STEC-infected faeces for subsequent faecal spiking experiments. The use of chilled, stationary O84:HNM stems from an evaluation of short pre-enrichments by Arthur et al (118), which emphasised the importance of matching STEC growth characteristics – most notably replicating the lag phase – that would be found in naturally contaminated samples. The physiological state of the target cells also has an effect on PCR efficiency (134). The stationary phase culture demonstrated that 600 nm OD reads using the O84:HNM OD curve could be used to dilute overnight cultures for spiking experiments without the strain’s death phase affecting the accuracy of the curve.

The faecal screening assays ensured candidate faecal specimens for spiking did not have endogenous STEC and acted as a cursory test of the culture conditions to be used for the main assays.

The faecal spiking experiment was conducted to determine the common limit of detection (LOD) of the STEC pathogenic genes in spiked faecal samples, as performed in similar studies (85, 116). As there is some conjecture regarding the effect of endogenous faecal E. coli on the sensitivity of PCR assays (85, 95, 102, 154), faecal samples β and γ, with a large difference in their endogenous E. coli populations, were chosen. The three pathogenic genes could be detected at 2 x10⁴ CFU per g faeces in TSB cultures of samples β and γ before incubation. This limit matches the capabilities of similar TaqMan PCR assays without enrichment (97, 100, 101). The LOD was consistent across the two faecal samples and with their exponential-phase equivalents, indicating that the sensitivity of the pathogenic gene assays were not affected by the levels of the endogenous E. coli nor the growth phase of O84:HNM. The pathogenic targets could be detected in samples inoculated with 2 x 10² CFU per g after 3 hours of incubation. The ability of the stationary, cold-stressed STEC to multiply that rapidly in faecal specimens
in 3 hours raised concerns that the OD curve was inaccurate and the spikes had not been
diluted properly. The following experiment, using plate cultures to confirm dilutions of
stationary O84:HNM based off the O84:HNM OD curve, verified that the dilutions were
accurate. The plate counts of the same culture after a 3 hour incubation indicated that
stationary-phase O84:HNM spent only approximately two *E.coli* generation cycles in lag
phase (108). In reviewing literature, the replication cycle time for STEC at 37°C was
found to be slightly shorter (18.1 minutes) than anticipated (approximately 20 minutes)
(108). It is therefore feasible that the STEC strain used could have multiplied to the level
of detection in the 3 hour period, but concerns persist that the laboratory-reared
O84:HNM may overestimate the performance of the incubation to resuscitate STEC
sourced from faecal samples (118, 155).

Hidaka *et al* makes reference to competitive primer testing for STEC genes (92). The
amplification of a gene target with a large concentration difference to others in the assay
(as with *uidA* in some samples) may influence the amplification of the targets with lower
concentrations. Future sensitivity testing would benefit from testing samples with
disproportionate concentrations to determine if this effect is negligible with the primers
used.

**3.4.5 ROX-inclusive master mix validation**

The attempt to substitute the no-ROX ToughMix with a readily accessible ROX-
inclusive qPCR mix was unsuccessful. A no-ROX ToughMix was recommended by the
distributor (DNature; personal communication) as some molecular “cross-talk” had been
experienced between ROX (λ = 610) and TAMRA (λ = 555) by other clients. Normally
the endogenous ROX dye functions as a baseline fluorescence for the normalisation of
other fluorescent signals and is incorporated to account for minor variations in plate
characteristics, volumes, or the quality of the optical system (88). As the algorithmic
correction problem could be circumvented, and as sensitivity of the assay was more
important than refining the reproducibility, it was decided to proceed with the ToughMix
qPCR mix. Testing of ROX-inclusive ToughMix was not an option due to time and
budgetary constraints, but exists as an option that could be explored for future studies.
Alternatively, probes that fluoresce at infrared wavelengths can be used to minimise the
influence of background fluorescence (86), but the platforms available for use with this
project were incapable of measuring fluorescence at this part of the spectrum.
3.4.6 384-well plate assays
Adapting the multiplex assay to 384-well plates did result in a decrease in fluorescence amongst test specimens, but the assay experienced no significant reduction in sensitivity. The first 384-well plate to be loaded and assayed appeared to have been underfilled. It remains unclear to the researcher what happened to this fluid, as the interval between dispensation by the robot and the covering of the plate with an optical seal (which would later form a hermetic seal on the heating block within the thermocycler) was not long enough to account for evaporation. No significant amount of ToughMix remained within the source microcentrifuge tubes on the platform. The only plausible explanation at the time was that the viscosity of the qPCR mix had caused the pipette tips to dispense erratically, and perhaps discarded the excess fluid into the run-off container on the platform. The subsequent cursory test of robot dispensation suggested the robot loader was capable of dispensing and mixing a viscous solution appropriately.

As for the loading of the reattempted plate, the underfilled wells on the right half of the plate occurred due to a build-up of airspaces in the source microcentrifuge tubes of the qPCR mix. As the wells on the right side were the last to be filled by the robot loader programme, a combination of qPCR mix viscosity and recurring airbubbles within the dispensing tips meant that tips would frequently dispense nothing to these wells. This second loading took place under direct observation. Increasing the number of dispensing tip changes (during the qPCR mix distribution step in the programme) did not prevent erratic dispensation. As there was no clear solution to ensuring that ToughMix-based solutions would be dispensed consistently by the loader, and due to budgetary constraints prohibiting any more extensive testing with ToughMix on 384-well plates, it was decided to revert to the use of 96-well plates, albeit with reduced volumes of reagents if feasible.

3.4.7 Results of main assays
The prevalence of stx and eae within samples sourced from Dunedin Hospital were found to be 1.53% and 4.41% (95% CI) respectively. The stx genes denote the presence of a subpopulation of Shiga toxin E. coli, while eae is a marker of the LEE mechanism, a characteristic feature of EPEC. As with other surveys of diarrheic stools (156-159), the presence of STEC or EPEC is indicative rather than conclusive of the aetiology of diarrhoea in each case, as the presence of both EPEC and STEC in asymptomatic subjects are a common occurrence (157, 160-162), the level of expression of the toxin
can be strain dependent (106, 152, 153), and diarrhoea may have non-infectious aetiologies. Nonetheless, the lack of other co-infecting pathogens in surveyed STEC samples (160, 162), and the strong association of the stx and eae genes with diarrhoea (22, 33, 50, 57), is suggestive that STEC and EPEC are the causative organism of diarrhoea in these patients. In reviewing the SCL microbiology test results for patients positive for stx genes, it was found that no aetiology had been reported. No other cause of diarrhoea was resolved for all but one of the patients that tested positive for eae (EPEC). There is currently no methodology in place at the Dunedin branch of SCL to detect EPEC. As the STEC prevalences determined are those for diarrhoeal stool samples, and as diagnostic laboratories that report to surveillance bodies do not test for all STEC (7), the STEC or EPEC prevalences in this project cannot be compared directly with population incidences as typically detailed in surveillance reports (13, 163). Moreover, it is inappropriate to compare project prevalences to regional STEC infection estimates as the Dunedin branch of SCL services regions outside that of Otago, including the Marlborough, Taupo, and Blenheim regions. Previous international studies reported the prevalence of STEC ranged from 1.2% - 3.49% amongst adults and children with diarrhoea (156, 159, 164), with higher rates usually seen in studies sampling children exclusively (158, 164, 165). As can be seen in Table 8 in the Results section, all of the STEC positive samples were sourced from patients ≥12 years of age. Although the overall STEC prevalence is comparable to other countries, the age group distribution of the stx positive samples is in contrast to international trends (43, 48, 156, 158, 159, 164, 165).

As many studies report the highest frequencies of STEC infection in children (2, 7, 36, 43, 48), it was expected that most or all of the positive samples would be from patients ≤12 years of age. The lack of STEC infection reported among children is unusual, but the milder course of non-O157 infection (21, 32, 36, 166), and the tendency to not seek medical attention for less severe infections may contribute to underdiagnosis in this age group (167). All of the non-O157 STEC samples were positive for stx2 and none were found to carry the stx1 toxin variant alone, contrary to the frequencies of these variants seen in European studies (21, 32, 168). stx1-only STEC infections are associated with more mild symptoms and uncomplicated diarrhoea and so may also be subject to underreporting (2, 33). It is also possible that the lack of stx1-only STEC samples reflects country-to-country strain differences, as stx1-only STEC strains have been isolated from
Australian HC and HUS sufferers (169), despite previous studies indicating Stx1 is much less toxic than Stx2 (5, 15).

An important factor to consider regarding these results is the seasonality of STEC infection. The highest rates of STEC isolation are typically encountered in the warm seasons of most countries (7, 10, 165, 170, 171). For Australia, this is roughly months December – February (10, 163, 169), whereas New Zealand generally experiences the highest rates in the months January – April (13, 72). The survey period for this project was 4th of July – 8th August, effectively the New Zealand winter, and a low period for the STEC infections reported to ESR in that year, as displayed in Figure 32. As such, the STEC prevalence reported for this study likely represents a baseline infection rate. The marked seasonality seen in New Zealand is attributed by some authors, as in other countries (163, 165), to increased consumption of undercooked meats during summer months (172). A recent New Zealand based study by Jaros et al, however, found no association of STEC infection with the consumption of contaminated foodstuffs, instead finding contact with pets, farm animals, manure and environmental water sources as important risk factors (72). Contact with animal manure is a leading risk factor for STEC infection in other countries (173, 174), and along with animal contact, is a commonly identified activity amongst surveyed STEC patients in New Zealand (13). Several studies allude to improved survival of STEC in cowpats, effluent, and on environmental surfaces in warmer ambient temperatures (63, 93, 175, 176), while others reference the increased shedding of STEC by cattle during summer months (35, 177, 178). It is likely that STEC infection seasonality is as a result of these factors acting in conjunction with increased outdoor activity during the New Zealand summer (72). It may be worthwhile testing the stx2, eae’ STEC samples with O104:H4 serotype-specific primers in case this prolific European strain is resident in New Zealand (55, 179).

An intriguing finding has been the discovery of a 6% prevalence of STEC infection amongst diarrhoeic samples undergoing testing for H. pylori. Faecal samples suspected of H. pylori infection by physicians are sent to SCL for enzyme immunoassay antigen testing (Premier Platinum HpSA© Plus), separate to the main panel of faecal tests. The samples set aside for H. pylori testing at SCL were prospectively tested for the presence of STEC and EPEC. H. pylori infection usually presents with symptoms of dyspepsia and reflux in association with gastric and duodenal ulcers (180, 181). A recent review of
Figure 32. STEC infections by month, January 2009 – December 2014, as published by ESR. STEC infections as reported to ESR generally have the highest incidence in the New Zealand summer and autumn months (February – May). The sampling period (4\textsuperscript{th} July – 7\textsuperscript{th} August 2014) for the project is shown between the red lines. It should be noted that although the reported data does establish New Zealand seasonality, the prevalences as reported by ESR cannot be directly compared to project findings as many laboratories do not employ molecular discovery of all STEC serogroups.

New Zealand primary care consultants found that many patients also present with symptoms of abdominal pain (182), and so it was theorised that an overlap in symptoms with that of STEC and EPEC infection may lead to improper testing. Chronic *H. pylori* infection is associated with gastric achlorhydria (183), and as this may improve survival of ingested STEC or EPEC transiting the stomach (105), the *H. pylori* samples were examined to determine if there was a significant rate of co-infection. A Fischer’s Exact probability test did not indicate that there were more STEC among *H. pylori* samples than encountered in other diarrhoeic stools (*p* = 0.063; 95% CI), but as there were only 50 surveyed *H. pylori* samples, the result is indicative that more extensive testing may establish significance. None of the samples that tested positive for STEC or EPEC were found positive by faecal *H. pylori* antigen testing, and a lack of published research has led the researcher to disregard co-infection with *H. pylori* having a significant role in STEC or EPEC infection. Put together, the *H. pylori* findings indicate that a small proportion of samples sent for *H. pylori* testing may be misidentified STEC infections.

As stated, the prevalence for *eae* was found to be 4.41% of the main pool of diarrheic stools surveyed. As EPEC is billed as a primary cause of infantile diarrhoea in developing countries (43, 161), it was expected that most of the samples positive for EPEC would be derived from patients ≤12 years of age. It was found that 15 of the 23 (65.2%) samples that tested positive for *eae* only were from patients ≥12 years of age, and that most of all *eae* patients were outpatients. It is difficult to infer, at least without more detailed patient information, exactly what has led to the infection demographics seen. While some studies do point to EPEC being associated with travel (184), EPEC is not implicated as a cause of traveller’s diarrhea to the same extent as enterotoxigenic *E. coli* (ETEC) in countries with high endemic rates of EPEC (43). As patient personal information remains confidential, the precise role of tourism or agricultural practice on EPEC demographics seen here remains somewhat notional. As the regions surveyed by the Dunedin branch of SCL are largely rural, and New Zealand cattle and sheep are significant reservoirs of STEC and EPEC (185), the most likely source of infection is direct contact or exposure to foods or water sources contaminated by bovine or ovine faeces (72). The variable rates of asymptomatic carriage of EPEC between countries (157, 161, 186-188), and the ability of EPEC to spread through person-to-person transmission (189), suggest that humans may also act as transitory reservoirs of endemic infection. Investigating EPEC epidemiology further would likely require in-depth region- or occupation-stratified
questionnaires and would benefit from an assessment of the prevalence of EPEC in healthy human stools.

As EPEC is a focus of childhood diarrhoea research, few studies exist that document the prevalence of EPEC in diarrhoeal stools sourced from both children and adults. Perhaps the most comparable prevalence data is that of an Australian study conducted by Staples et al in 2013, which returned an EPEC prevalence of 6.8% from mostly diarrhoeal stools collected throughout Queensland from 2008-2011 (190). The highest prevalence of EPEC in this study was recorded amongst children <5 years of age (17.2%). A 2001 Finnish study by Keskimaki et al reported an EPEC prevalence of 2.3% amongst diarrheic patients (54). A study in Beijing by Qu et al reported a prevalence of 0.9%, a prevalence less than that of other enteric pathogens such as Salmonella and Shigella (171). Another study in Germany of diarrhoeic and non-diarrhoeic stools reported a prevalence of 8.9%, greatly exceeding that of other enteric pathogens (184). The prevalence of EPEC in children varies considerably from country to country (2.8 – 27%) with differing rates of asymptomatic carriage (157, 186-188, 191, 192). The highest rates of EPEC infection occur in the warm seasons of countries (171, 187), although seasonality appears to be less marked for EPEC in younger children (193).

In reviewing the SCL microbiology test results for patients positive for EPEC, it was found that very few samples had an aetiology for the diarrhoea determined. Although EPEC is known to have high rates of co-infection with other pathogens in children (157), the extensive faecal pathogen testing conducted by SCL (for a list of pathogens tested for by the Dunedin branch of SCL, refer to Table 6) is consistent with EPEC being the likely causative agent of diarrhoea in most of these patients. Sorbitol non-fermenting colonies were identified on CT-SMAC from 3 of the eae positive samples, which were confirmed as E. coli by MALDI-TOF analysis (refer to Figure 6 in the introduction) before being sent to ESR for Shiga toxin testing. All 3 samples were recorded as testing negative for Shiga toxin by ESR. As most EPEC strains are sorbitol fermenting (43, 74), it is likely SCL is sending irrelevant E. coli isolates to ESR in cases of EPEC infection. These findings do not render the use of SMAC-based media to detect O157:H7 redundant, but the prevalence of EPEC, in conjunction with the rates of sorbitol non-fermenting STEC in diarrhoea, suggest that an update of diagnostic algorithms is needed to meet the more salient causes of diarrheic disease in New Zealand. An important consideration for future
studies is the bundle forming pilus (bfp), encoded by the *bfpA* gene (194). The bfp is an accessory, aggregative colony-forming attachment factor in EPEC and commonly used to differentiate ‘typical’ from ‘atypical’ subsets of EPEC (43, 161). Typical EPEC was considered the leading cause of diarrhoea in previous decades, but recent data suggests atypical EPEC may now be more prevalent in the developed and developing world (161), with reports of outbreaks and endemic disease in both (195, 196).

There were too few faecal calprotectin samples (*n* = 26) to draw a meaningful comparison of the rate of EPEC in these samples with that of regular diarrhoeic specimens (*p* = 0.298827; 95% CI). Calprotectin is a neutrophil cytosolic protein released at increased concentrations during active bowel inflammation, and is one of the primary markers used to discriminate IBD from irritable bowel syndrome (197). The faecal calprotectin samples were investigated as severe STEC infection can mimic IBD clinically (27), and faecal calprotectin levels may become elevated in infection with EHEC (15). The diagnostic algorithm for IBD at SCL involves an immunochromatographic screening assay (CERTEST Biotec Calprotectin). Samples suspected of IBD that tested positive with this test were confirmed with a quantitative test that detected faecal calprotectin levels of >50 mg/g faeces. The two *eae*+ samples tested negative by the quantitative test. This indicates EPEC may have had some minor role in raising the calprotectin levels, but it is difficult to surmise more of the role of this pathogen in these patients.

### 3.4.8 PVPP column testing

Of the 522 samples assayed with the multiplex PCR, 95 samples showed either very low levels of amplification (Ct over 38 cycles) or no amplification of *uidA* whatsoever, possibly reflecting the effect of an inhibitory compound not alleviated by the pre-enrichment dilution or Chelex (198). The promotional material for perfeCTa™ ToughMix stated the qPCR mix to be resistant to the inhibitory effects of polyphenols, humic acids, haemoglobin, haematin, and polysaccharides. Chelex, with affinities to chelating Cu²⁺, Zn²⁺, Ni²⁺, Cd²⁺, Pb²⁺ and Fe²⁺ ions, was reasoned to have a complementary effect in removing inhibitory heavy metals from samples (124, 140). The PVPP, BSA and plasmid experiments were conducted to prospectively identify post-extraction techniques that could remove putative inhibitors. PVPP spin columns were initially investigated as they were demonstrated to be effective in removing humic and phenolic compounds from soil and bovine faeces and were available for purchase.
separate to DNA isolation kits (116, 199, 200). Humic acids and polyphenols were considered as candidate inhibitors that might have avoided elimination due to varying amounts in human faeces (as derivatives of degraded plant matter) (124, 200) and the variable water solubility of humic acids (199). Preliminary testing with PVPP spin columns indicated that amplification within a significant proportion of the faecal samples could be remedied. However, the amplification of only a small number of faecal samples was improved when PVPP processing was applied to the remainder of samples showing poor amplification. A few instances of a slight decrease in DNA yield were observed after PVPP processing, consistent with the findings of similar studies conducted with bovine faeces and soil (124, 199). Although BSA is recommended in several PCR studies to counteract the inhibitory effects of lactoferrin and haemoglobin, as may be encountered in faecal samples with occult blood (88, 124, 198, 201), the addition of BSA resulted in no significant improvement in amplification. It is likely that the addition of BSA is redundant; the Fe$^{2+}$ chelating properties of Chelex would assist in removing iron containing compounds (140).

Subsequent testing of specimens that remained negative with an internal amplification probe revealed that the lack of uidA was not as a result of active inhibition, and that PVPP had no significant effect in improving DNA yield. The decision to assess diarrhoeic stools may have helped in this regard; diarrhoeic stools are postulated to have less potentially inhibitory anaerobic and dietary material as a result of the shortened colonic transit time (202). The most plausible explanation for the lack of amplification in the absence of PCR inhibition is that \textit{E. coli} in those samples were either absent or the number was below the limit of detection of the multiplex even after the 3 hour TSB pre-enrichment. \textit{E. coli} are recognised to be a small and sometimes undetectable fraction of enteric flora (111, 203), but the significance of the lack of uidA amplification is difficult to determine as there are few published measurements of \textit{E. coli} CFU g$^{-1}$ faeces ranges. Bailey \textit{et al} notes several studies of healthy subjects where \textit{Enterobacteriaceae} and \textit{E. coli} CFU counts range from $1 \times 10^4$ to $1 \times 10^9$ CFU g$^{-1}$ of faeces (203), while London \textit{et al} noted 28% of tested faecal samples had $<10^3$ \textit{E. coli} CFU g$^{-1}$ in plate culturing (204). As seen in the stationary-phase experiments, stationary-phase, cold-stressed STEC growth rates in faecal samples were not greatly hindered. It is possible that \textit{E. coli} in some naturally infected faeces do not share the growth characteristics of the spike model due the action of bacterial colicins or other enteric stressors (81, 145, 155, 205-207). Antibiotic regimens of the patients
involved in the study remain confidential, and so the role of antibiotic suppression of *E. coli* growth for any samples remains a point of speculation. It is also possible that *E. coli* strains may become ‘conditioned’ to survival within faecal matrices. Ibarra *et al* (30), describes an enhanced pathogenicity among EHEC strains isolated from faecal specimens compared to their immediate parent strain (208); it is not unreasonable to suppose that similar conditioning may result in an adaptive stunted growth of STEC in short-phase culture, but further experimentation would be necessary to support this theory.

Any investigation of the properties of the *E. coli* strains supposedly missed by the enrichment-extraction method chosen for the project would likely require a comparison with a more sensitive method. A comparative study recently conducted by Wagner *et al* (136) highlighted a number of potentially more sensitive (albeit more expensive) faecal DNA extraction kits that could be used to recover ‘missed’ strains, perhaps combined with a longer pre-enrichment phase. As Chelex is only a metal-chelating resin, biases introduced by proteins and other particles limit the accuracy of spectrophotometric quantification or standardisation of the DNA of Chelex-extracted samples (131). Qubit-based fluorometric DNA quantification of faecal DNA is one avenue that could be explored if future experimentation with Chelex is pursued (209). *E. coli* markers other than *uidA* such as *wecA* could also be explored (62), although the failure of this assay is unlikely as all *E. coli* controls to date have performed as expected. Ultimately, there could be many reasons for the lack of *uidA* amplification seen, but these proposed experiments could help to resolve details.

### 3.4.9 LAMP based detection of O157 serotypes and ESR serotyping

The LAMP analysis of the *stx* positive samples indicated that the primers used were capable of delineating O157 serogroups of STEC from non-O157 serogroups. The LAMP results were later verified by molecular serotyping performed by ESR. The results indicate a basic LAMP assay could be incorporated as an inexpensive part of a diagnostic algorithm (90), or incorporated as a confirmatory test of O157 serotypes in multiplex-based screening.

The serotyping results returned by ESR specified that all but two of the *stx*+ samples could not be serotyped. This is in contrast to international STEC trends; most isolates of
non-O157 *E. coli* that are associated with human disease belong to the serogroups O26, O103, O145, O111, O45 or O121 as would be detected by the ESR *wzx* serotyping assays (21, 32, 36, 169, 210). Comprehensive serotyping of the non-O157 *stx*+ samples was not possible as the samples were retained in extracted form, and few serotype-specific primers for other the more obscure serotypes have been developed. A study of stool specimens at Dunedin Hospital in 2001 by Brooks *et al* revealed a variety of unusual non-O157, non-‘big six’ serotypes, with no clearly dominant serotype (211), and a study by the same author in 1997 encountered some overlap in serotypes recovered from meats sourced from Dunedin retail outlets (212). Similarly, a study of clinical specimens sourced from medical laboratories and the Enteric Laboratory at ESR by Jaros *et al* (72), found a host of non-O157 *stx*+ serogroups, many of which are also isolated from New Zealand sheep and cattle (70). Notably, the genomic DNA profiles of O84:H- isolates derived from New Zealand cattle were determined to be sufficiently similar to that of clinical isolates by PFGE analysis as to infer medical significance (71). It is likely that the non-O157 specimens extracted in this project belong to a combination of the serotypes listed in these studies. An Australian analysis of state and national STEC infections by Vally *et al* (10), albeit likely influenced by SMAC-based reporting (213), reported 58% of serotyped STEC infections being of the serogroup O157, with a further 13.7% of serogroup O111 and 11.1% of serogroup O26.

The two samples found to be positive for the serogroups O157 and O103 are an intriguing find. While co-infections involving two or more STEC serotypes are not unusual (214), two instances of infection with the same two STEC serogroups are somewhat suggestive of a common source infection or transmission event. The inability of the ESR *fliC* assay to determine a flagellar (H) antigen in these two samples is also surprising; this suggests that the O157 strain seen in these samples may belong to one of the rarer and generally less pathogenic O157 serotypes, such as O157:H6, O157:H39, O157:H45, or O157:H16, among others (215, 216). These non-H7 O157 types are noted to have high rates of sorbitol fermentation (217). Another plausible explanation, and one that fits well with the samples being severe enough to warrant clinical attention and yet remaining undetected by conventional methods, is that the H antigen in the observed O157 strains is untypeable, and therefore possibly the O157:H- serotype. O157:H-, or O157:HNM (non-motile) is a unique STEC serotype first isolated in a 1988 outbreak in Germany (218, 219). Approximately 10% of O157 infections and HUS cases are caused by
O157:H- strains in continental Europe (75, 210, 220), and the serotype is recognised to have high rates of tellurite sensitivity and sorbitol-fermentation (75, 98, 210, 221). As a result, this serotype is inhibited by tellurite used in CT-SMAC and indistinguishable from commensal enteric bacteria on unsupplemented SMAC (75, 98, 221, 222). All European isolates of O157:H- strains express high levels of Stx2 toxin (210, 223), and the clinical profile of the serotype is not considered to differ significantly from O157:H7 in paediatric patients (220). A third possibility that cannot be dismissed, and one that also applies to the other stx+ and eae+ samples, is that other untyped stx- or eae-carrying E. coli strains exist within these samples, and that multiple strains may be present and in possession of combinations of the detected genes. One of the limitations of the multiplex assay used in this project is that the origin of the virulence factors cannot be determined in cases of STEC co-infection, so it is impossible to determine whether the stxi and eae detected in the two O157:H- samples were derived from the O157:H- strain or the co-residing O103 strain, or a third party E. coli. Ultimately, this is a shortcoming of the use of multiplex qRT-PCR; live isolates of these samples would be required to determine more detailed serological information and comprehensively establish the origin of each virulence factor in each sample.

One point of consideration for future studies or in-depth profiling of the existing extracted stx2+ samples are stx2 subvariants. The stx2 assay used in the project multiplex does not discriminate between the stx2 subvariants stx2, stx2c and stx2d, which are noted to have differing associations to severe disease and progression to HUS (34, 224). A finer characterisation of stx2+ STEC strains could be undertaken with the use of stx2 subvariant-specific primer sets (34, 53, 210, 224). The use of SK-LP primers to characterise intimin subvariants could also be employed in future studies (218). Determination of virulence profiles of O157:H- extracts may provide information concerning the geographical origin (European or Australian) of these organisms. A larger survey than the one reported here would establish whether the O157:H7 or O157:H- serotype was dominant in diarrhoeic samples received by SCL at Dunedin Hospital.

3.5.0 Conclusions and future considerations
A multiplex qRT-PCR assay capable of detecting marker genes for STEC and EPEC was successfully adapted for use in high-throughput diagnostic practice. The protocol was developed to include a 3 hour non-selective TSB pre-enrichment to boost the sensitivity
of STEC and EPEC detection and use of Chelex-100 resin as an inexpensive and labour-efficient DNA extraction method. It was found that the protocol could not be adapted to derive meaningful quantitative information on the levels of STEC or EPEC bacteria in infected stool samples. The pre-enrichment was demonstrated to boost sensitivity to 2 x 10^2 CFU per g faeces in faecal STEC growth models. The prevalence of STEC and EPEC among diarrhoeic stools sourced from SCL, Dunedin Hospital was determined to be 1.53% and 4.41% (95% CI) respectively. STEC were found in several faecal samples set aside for H. pylori testing, and trends indicate a significantly higher rate of STEC infection may be found among these sample compared to the rest of the diarrhoeic stools in a larger survey (Fisher Exact test (p = 0.063; 95% CI). The multiplex assay was also used to screen samples set aside for IBD testing, and although no statistically significant results were found, there was evidence implicating EPEC in raising faecal calprotectin levels in some patients and contributing to redundant isolates being sent to ESR. Testing of LAMP-based amplification revealed some applicability to diagnostic practice in being able to discriminate O157 serogroups from non-O157s. Commercial molecular diagnostic systems, such as the real-time Enteric Bio Gasto 2 (SeroSep Ltd, Annacotty, Ireland) include stx1 and stx2 testing panels (225). However, these systems are not designed to detect intimin, which is a marker for EPEC, and a marker of increased virulence for STEC. The qRT-PCR assay developed in this project was able to detect the gene markers for both STEC and EPEC, the latter being more abundant in the diarrhoeal stool samples tested in this project.

A number of directions were considered for future research into the role of STEC and EPEC in human health in New Zealand. Further investigation into the prevalence of STEC and EPEC would benefit from study in the months of peak infection in New Zealand, likely including a culture-based component so that more extensive serotyping information can be derived from live isolates. A culture based component to testing would also help to determine which strains are dead or non-viable but detectable by PCR (150, 151). If Chelex-based DNA extraction is to be pursued for faecal samples in diagnostic practice, protocol modifications and the use of Qubit could be implemented to improve extraction consistency (141, 209, 226, 227), and acid-pretreatment could be explored to improve assay specificity without compromising the effects of short pre-enrichments (105). A more detailed examination of the stx2 and intimin subtypes and other virulence factors in the existing extracted samples or other surveys could provide
closer links to exact pathologies seen in the literature (34, 55, 179, 210, 224). Ultimately, the prevalences of STEC and EPEC uncovered by this project are indicative of an underappreciated role of these pathogens in New Zealand diarrhoeal disease.
References


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Appendices

Appendix 1

Standard Operating Procedure for the use of STEC and faecal samples

1. Purpose:

The intent of this study is to determine the prevalence of non-O157 serotypes of Shiga-toxigenic *E. coli* (STEC) in diarrhoeal faecal samples collected by Southern Community Laboratories.

The experimental procedure is expected to comprise liquid media culturing and DNA amplification of selected STEC strains as a preface to testing faecal samples of unknown pathogenicity.

2. Hazards, Exposures, Controls, and Containment:

The primary hazards in initial testing will be *E. coli* strain serotypes O111, O91NM, O130H11, O84 (O111 obtained from Brooks lab, Shiga toxin-carrying O91NM, O84 and O130:H11 obtained from ESR). These pathogens may be transmitted through ingestion or contact with the mucous membranes of the mouth, nose and eyes, and are recognised to be further spread through the contamination of work surfaces and fomites. Symptoms of exposure to *E. coli* range from mild, watery diarrhoea to abdominal cramping and bloody diarrhoea. If you contract these symptoms, notify your supervisor immediately.

Subsequent testing will involve faecal samples likely to contain a variety of gastrointestinal pathogens, most notably Hepatitis A, Norovirus, *Cryptosporidium*, and other pathogenic strains of *E. coli*. The use of faecal samples represents a significant health risk as some faecal pathogens (particularly Norovirus) have extremely low infectious doses, can be spread through inhalation of aerosolised emesis, and are resistant to some forms of disinfection. Refer to Table 1 for the principle hazards of faecal samples.

All laboratory work with live *E. coli* strains O91NM, O130H11, and untreated faecal samples will be undertaken in a Class II biosafety cabinet. The use of racks and other holders is recommended to limit spills inside the cabinet. Enteropathogenic *E. coli* O111 is shiga-toxin negative and may be handled on the bench, providing appropriate PPE is used. Any work with O111 likely to generate aerosols, (eg. vortexing) must be performed inside the cabinet.

Barrier tips will be used for pipetting work with *E. coli* strains and faecal samples.

All laboratory workers must wear appropriate Personal Protective Equipment (PPE; lab coats and disposable gloves). Double-gloving and the use of disposable lab coats or sleeves are recommended for the processing of faecal samples. Change/decontaminate outer gloves and disposable coats/sleeves frequently and in the event of spills on gloves/sleeves to avoid contamination of lab surfaces. Decontaminate culture vessels, secondary containers and other
equipment before removing from Class II cabinets, especially the interior of pipettor barrels. Disposable PPE must be discarded into marked biohazard bags and lab coats must be removed before leaving the lab. Outer gloves and disposable sleeves should ideally be removed and disposed of into discard containers inside the cabinet following the decontamination of all equipment and surfaces. Be sure to change gloves or sleeves if you suspect they are contaminated.

Properly extracted DNA from the sample *E. coli* and faecal samples poses negligible transmission risk and so does not require use of the biosafety cabinet, but PPE is still recommended.

Wash hands thoroughly upon leaving the lab.

Where possible, but particularly in the event samples need to be transported outside the laboratory, samples must be contained in sealable secondary containers (click-clacks). Include absorbent material in the secondary containers to soak up any spilled material (change as necessary).

3. Experimental procedures

The *E. coli* serotypes and faecal samples will be cultured, incubated and chilled with media designed to amplify *E. coli*. The use of secondary containers for liquid/faecal sample incubation/refrigeration is recommended, although parafilm wrapping will be sufficient for the refrigeration/incubation of plate cultures. Cultures and containers with potentially infectious organisms will be labelled with appropriate biohazard stickers/laminated labels.

4. Inactivation, Decontamination, Waste Treatment and Disposal

All work surfaces including the biohazard cabinet must decontaminated before and after use of live species and faecal samples, at the end of a working day, and immediately after any spills. A 1:10 dilution of household bleach (made fresh daily) with 5-10 min contact time is the preferred disinfectant, though others may be useable. Hydrogen peroxide (for the inactivation of Cryptosporidium) is itself partially inactivated by bleach, so an excess of bleach must be used following hydrogen peroxide treatment of spills, and the use of hydrogen peroxide is only recommended in the event of a spill of a faecal sample. It is not necessary to clean the cabinet surfaces with hydrogen peroxide as the cabinet UV light is sufficient in killing *Cryptosporidium* (see Table 1). As bleach is corrosive, a final ethanol wipe down must be performed to preserve the integrity of the metal surfaces in the cabinet.

Outer gloves and sleeves must be removed/changed after work in the cabinet.

Must take care to avoid contamination of pipettors and other equipment inside the cabinet. All used equipment and unused samples must be decontaminated or disposed of in red biohazard bags.

5. Spills and Emergency Exposure Procedures
In the event of spills outside the cabinet, cover any spills of live bacteria with paper towels. Saturate towels with 1:10 dilution of household bleach (made fresh daily) and allow 5-10 min contact time. Wipe up area and discard towels in red biohazard bag. Repeat disinfection procedure until surface is clean. Wash any exposed skin thoroughly with soap and water. Perform first aid if necessary. Along with supervisor, complete accident report form.

<table>
<thead>
<tr>
<th>Infectious Dose</th>
<th>Transmission</th>
<th>Incubation Period, Duration</th>
<th>Symptoms</th>
<th>Recommended Surface Disinfectants</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norovirus</td>
<td>&lt;10 virus particles</td>
<td>Person-to-person, fecal-oral, fomite contamination, aerosols.</td>
<td>12-48h, up to 60h.</td>
<td>Acute onset projectile vomiting, non-bloody diarrhoea, abdominal cramps. Self-limiting.</td>
<td>1000-5000ppm chlorine bleach</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>Unknown, but likely 10100 virus particles</td>
<td>Fecal-oral, close personal contact.</td>
<td>15-50 days (median 28 days), duration variable.</td>
<td>Nausea, vomiting, diarrhoea, dark urine, jaundice, fever, headache, abdominal pain. Self limiting.</td>
<td>1:100 bleach</td>
</tr>
<tr>
<td>Cryptosporidium sp.</td>
<td>~30 oocysts, theoretically may be as low as 1</td>
<td>Fecal-oral, either directly or via fomites. Respiratory infection possible but rare.</td>
<td>5-9 days, 11-13 days.</td>
<td>Self limiting watery diarrhoea, abdominal cramps, vomiting, fever.</td>
<td>Noted to be resistant to chlorination; 6% hydrogen peroxide is effective. UV light will disinfect cabinet surfaces. *</td>
</tr>
<tr>
<td>E. coli O157</td>
<td>&lt;50 organisms</td>
<td>Fecal-oral or person-to-person, directly or via fomites.</td>
<td>2-10 days (2-3 day median), 1 week.</td>
<td>Bloody diarrhoea, abdominal cramps. May have fever, vomiting. May progress to HUS, hospitalisation.</td>
<td>1:100 hypochlorite bleach, 70% ethanol</td>
</tr>
</tbody>
</table>

* At current wattage (740mW/m²), the biosafety cabinet UV light can inactivate >90% of Cryptosporidium oocysts on cabinet surfaces within 90 seconds, based upon a lethal dosage threshold of >2.5mJ cm⁻² for C. parvum (8). Typical run time of the UV light following work is ~30mins.

Table References


http://www.cdc.gov/norovirus/preventing-infection.html

http://www.cdc.gov/norovirus/hcp/clinical-overview.html
Appendix 2

This document is a copy of a clarification requested by the University of Otago’s Human Ethics Committee regarding privacy concerns about the project, and contains relevant supplementary legal and ethical information. Written confirmation to access of faecal samples by SCL (as mentioned in this document) is not included, but was obtained prior to the Committee’s approval (and a requisite of it).
A copy of the Committee’s approval is provided at the end of this document.

The Committee request comment and clarification on the following:
1. Are the samples already held by Southern Community Laboratories (SCL), or will they be collected in the future. If the former, did the specimen-providing individuals give permission for future research to be conducted? If the latter, what grounds are there not to obtain informed consent?

The samples are to be collected by SCL in the future, pending Human Ethics Committee approval. Both the Human Tissue Act 2008 and Health Information Privacy Code 1994 can be seen to provide reasonable grounds to not seek informed consent for the purposes of this study, insofar as faeces are noticeably not mentioned as human tissue in the Act and no information that could be used to identify the patient will be supplied to the researchers in accordance with the Code.

In the case of the Human Tissue Act 2008, while section 7 (1a and 1b) does define human tissue as that being derived from a living individual and which includes human cells, no mention of faeces is made in the examples that follow in Section 7, despite urine, sputum, mucus and similar body fluids being clearly specified. It should be made clear at this point that the test to be performed is specific to E. coli and has no capacity to reveal information about the patient beyond the presence of Shiga-toxigenic E. coli bacteria in the stool sample.

Informed consent for the use of faecal culture inoculants is deemed unnecessary as defined by the Health Information Privacy Code 1994, Rule 11 (2) which states compliance with Rule 11 (1) (b) (that is, informed consent of the individual supplying the tissue sample) is not necessary if the information is (as is the case with this study) “(i) to be used in a form in which the individual concerned is not identified; or (ii) is to be used for statistical purposes and will not be published in a form that could reasonably be expected to identify the individual concerned; or (iii) is to be used for research purposes (for which approval by an ethics committee, if required, has been given) and will not be published in a form that could reasonably be expected to identify the individual concerned”. Any of the above clauses could be reasonably applied to the use of personal information in this study, although it is to be made clear that no information that can be used to identify patients supplying faecal inoculants will be released by SCL to researchers undertaking the project. As the study is intended to be a retrospective prevalence study of Shiga-toxigenic E. coli (STEC) and not expected to be of direct therapeutic benefit to the patients involved (detection of STEC is unlikely to be influential in the course of treatment, as the disease is usually of short duration and self-limiting), informed consent is deemed to be of little benefit. It should also be noted that, as SCL has a large and disparate sample catchment area, it is expected that seeking informed consent for the use of any and all samples is not practicable and that any
resulting shortcomings in patient response will have a deleterious effect on the relevance of this project as a prevalence study.

2. The answer in Section 4 of the form notes that there will be no access to identifiable health information. Presumably the stool samples will be initially identified at SCL. Please indicate what process will be used to de-identify the specimens. Samples will be used to inoculate enrichment broths which will only be labelled with patient status (in/out patient), age (whether ≤12 years or older only), and a unique ID assigned by SCL (not a personal identifier eg NHI number, but a special code determined by SCL for this research purpose). The intent is that the specimens received by the university are not the faecal samples themselves complete with personal information but derivative cultures of faecal materials with personal identifiers removed.

3. The Committee request comment on how abnormal results will be dealt with and how this will be communicated to the individual whose sample has been tested; if you are proposing to not inform individuals then the committee request a justification for not doing so.

The test used is a molecular-based assay specifically designed to only detect the presence of STEC and has no capacity to determine any other underlying health problems or causes of diarrhoea. Owing to the large number of specimens that are expected to be processed, the amount of time that this will take, and the cost-efficiency of testing individual samples, it is expected that for the purposes of this study the specimens will be analysed in large batches following processing, and that this will likely take place after any positive result will be of therapeutic value to health workers or the patients from which the samples were derived. Added to this, as the test in its current state cannot distinguish between the exclusively pathogenic serotypes of STEC and those with more tenuous links to human disease, it cannot be reasonably inferred that STEC is necessarily the causative agent of the diarrhoea in positive samples.

4. In Section 6, “N/A” is the response to the requirement for authorisation by the Health agency. If the health agency is providing the specimens it is authorising their use and the University requires formal authorisation.

A notice of formal authorisation for the project researchers to access faecal inoculants (as described in (2)) has been provided by SCL and is included with this documentation.
Appendix 3

Human Ethics Committee (Health) Departmental Approval of Projects using Health Information

Read the following guidance notes carefully to assess whether your study falls within the definitions as outlined in Part A or Part B below before completing this form. Should Part A or Part B apply to your proposed study then full ethical approval is not required as per the Health Information Privacy Code Rule 11 (2).

A  The use of unidentified data

You may complete this form if you are confirming that your study only uses information that cannot be linked to an identifiable individual (see the Health Information Privacy Code, Rule 11 (2) (c) (i))

Or

B  The use of identified data

You are using identifiable data that complies with the following definitions.

1. Your study is an Audit of Health Provision Process, or Outcomes, using health information as defined in Section 1 of the Health Information Privacy Code 1994 (see guidance notes (ii))

   AND

2. You have appropriate authority from the data holder to access health information (see question 6)

   AND

3. a. You are seeking informed consent from those whose personal information will be accessed. In this case, attach an Information Sheet and Consent Form.

   OR

   b. You are not seeking informed consent but personal information will not be published in a form that could reasonably be expected to identify the individual concerned. (see the Health Information Privacy Code Rule 2 (g) (iii))

If Part A or Part B applies to your proposal, please submit the form below with copies (if applicable) of an Information Sheet or Consent Form for participants immediately after it has been signed by the Head of Department to the Human Ethics Committee (Health), via:

Gary Witte (Manager, Academic Committees), Jane Hinkley (Academic Committees Administrator), or Jo Farron de Diaz (Research Ethics Administrator) Academic Committees Office, Rooms G22, G23 or G24, Ground Floor, Clocktower Building. A signed form can be emailed to either gary.witte@otago.ac.nz jane.hinkley@otago.ac.nz or jo.farrondediaz@otago.ac.nz

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1. Details of Investigators

Principal Investigator (University of Otago staff member responsible for project)

Name: Dr. Heather Brooks
Department/School: Microbiology and Immunology
Email: heather.brooks@otago.ac.nz

Co-investigators

Name: Rory O'Brien
Department/School: Dell Research Laboratory
Email: Rory.O'Brien@otago.ac.nz

Student Investigator

Name: Rizwan Thomas
Department/School: Microbiology/Immunology
Email: Rizwan.Thomas@otago.ac.nz

Level of Study: Masters

Where there are more co-investigators or student investigators, insert details on a separate sheet.

2. a) Title of Study: Molecular detection of non-O157 STEC in clinical stool samples.

b) Briefly and in plain English describe the background for the proposed study:

The aim is to develop and use a real-time PCR diagnostic assay to examine the prevalence of non-O157 Shiga toxin-producing (STEC) in stool samples sourced from SCL laboratories.

3. Funding Body/Sponsor

The sponsor is the organisation with overall responsibility for the initiation, management and financing arrangements of a study.

Which of the following best describe the sponsor(s) of your study?

☐ University of Otago ☐ another academic institution
☐ collaborative research group ☐ district health board (DHB)
☐ other government agency ☐ pharmaceutical company
☐ medical device company ☐ other (e.g. non-governmental organisation (NGO), or contract research organisation)

Please specify:

4. Does your study include access to Health Information where individuals are identifiable (see guidance notes, point (i))?

☐ Yes – go to question 5

☐ No

☐ Yes – go to question 5

5. If your study seeks access to identifiable health information will you be seeking informed consent?

☐ Yes – provide a Participant Information Sheet and Consent Form (templates can be found on the Human Ethics web page) – go to question 6
☐ No – If No - From your audit, will you publish any health information in a form which could reasonably be expected to identify any individual?
☐ No
☐ Yes - If YES a full University of Otago Human Ethics (Health) application is required with a full justification and protocol. The Application Form can be found on the University of Otago Human Ethics web page. On the full application you will need to: 1) Justify why this information should be disclosed to you, specifying the stipulations in Rule 11 of the Health Information Privacy Code 1994 which would allow disclosure without consent and/or 2) Confirm that the results of the study will not be considered for publication, other than within the health agency holding the information (e.g. in a peer-reviewed journal); if not, sign this form.

6. Where Health Information is being accessed:
   a. the Health Agency* where the information is held must provide authorisation allowing you to access the Information. Attach a signed authorisation to this form.
   b. a confidentiality agreement* is required confirming that patient confidentiality will be maintained at all times. Attach agreement to this form.
   * see guidance notes (ii & iii)

7. The University of Otago has a Policy for Research Consultation with Māori. Have you already completed, or do you propose to undertake Māori consultation? (Please see http://www.otago.ac.nz/research/maoriconsultation/index.html).
   ☐ yes, we have ALREADY undertaken consultation (attach a copy of your completed Research Consultation with Māori Form)
   ☐ no - if no, provide a brief outline of reasons why not

8. Signatures
   By signing here you are confirming that you have read the Health Information Privacy Code, specifically Rule 10 Limits on Use of Health Information and Rule 11 Limits on Disclosure of Health Information and that your proposed research complies with both Rules.

   Applicant’s signature: [Signature]
   (Principal Investigator)

   OR

   By signing here, you are confirming that your research does not fall within the definition of “Health Information” as defined by the Health Information Privacy Code (1994)

   Applicant’s signature: [Signature]
   (Principal Investigator)

   Name: (please print) [Name]

   Date: [Date]
☐ No – If No - From your audit, will you publish any health information in a form which could reasonably be expected to identify any individual?

☐ No

☐ Yes - If YES a full University of Otago Human Ethics (Health) application is required with a full justification and protocol. The Application Form can be found on the University of Otago Human Ethics web page. On the full application you will need to: 1) Justify why this information should be disclosed to you, specifying the stipulations in Rule 11 of the Health Information Privacy Code 1994 which would allow disclosure without consent and/or 2) Confirm that the results of the study will not be considered for publication, other than within the health agency holding the information (e.g. in a peer-reviewed journal); if not, sign this form.

6. Where Health Information is being accessed:
   a. the Health Agency* where the information is held must provide authorisation allowing you to access the information. Attach a signed authorisation to this form.

   b. a confidentiality agreement* is required confirming that patient confidentiality will be maintained at all times. Attach agreement to this form.

   * see guidance notes (ii & iii)

7. The University of Otago has a Policy for Research Consultation with Māori. Have you already completed, or do you propose to undertake Māori consultation? (Please see http://www.otago.ac.nz/research/maoriconsultation/index.html).

   ☐ yes, we have ALREADY undertaken consultation (attach a copy of your completed Research Consultation with Māori Form)
   ☐ no - If no, provide a brief outline of reasons why not

8. Signatures
   By signing here you are confirming that you have read the Health Information Privacy Code, specifically Rule 10 Limits on Use of Health Information and Rule 11 Limits on Disclosure of Health Information and that your proposed research complies with both Rules.

   Applicant's signature:
   (Principal Investigator)

   OR

   By signing here, you are confirming that your research does not fall within the definition of 'Health Information' as defined by the Health Information Privacy Code (1994)

   Applicant's signature:
   (Principal Investigator)

   Name: (please print)

   Date: 19.3.14
Appendix 4(a)

Ngāi Tahu Research Consultation Committee
Te Komiti Rakahau ki Kai Tahu

Wednesday, 19 February 2014.

Dr Heather Brooks,
University of Otago,
DUNEDIN.

Tētū Koe Dr Heather Brooks,

Molecular detection of non-O157 STEC in diarrhoeal stool samples.

The Ngāi Tahu Research Consultation Committee (The Committee) met on Wednesday, 19 February 2014 to discuss your research proposition.

By way of introduction, this response from The Committee is provided as part of the Memorandum of Understanding between Te Rūnanga o Ngāi Tahu and the University. In the statement of principles of the memorandum it states “Ngāi Tahu acknowledges that the consultation process outline in this policy provides no power of veto by Ngāi Tahu to research undertaken at the University of Otago”. As such, this response is not “approval” or “mandate” for the research, rather it is a mandated response from a Ngāi Tahu appointed committee. This process is part of a number of requirements for researchers to undertake and does not cover other issues relating to ethics, including methodology they are separate requirements with other committees, for example the Human Ethics Committee, etc.

Within the context of the Policy for Research Consultation with Māori, the Committee has consultation on that defined by Justice McGechan:

"Consultation does not mean negotiation or agreement. It means: setting out a proposal not fully decided upon; adequately informing a party about relevant information upon which the proposal is based; listening to what the others have to say with an open mind (in that there is room to be persuaded against the proposal); undertaking that task in a genuine and not cosmetic manner. Reaching a decision that may or may not alter the original proposal."

The Committee considers the research to be of importance to Māori health.

The Committee suggests researchers consider the Southern District Health Board's Tikaka Best Practice document, in particular patient engagement. The document also covers the collection, storage and disposal of blood and tissue samples. This document is available on the Southern District Health Board website.

The Committee suggests dissemination of the research findings to Māori health organisations regarding this study.

We wish you every success in your research and the Committee also requests a copy of the research findings.

This letter of suggestion, recommendation and advice is current for an 18 month period from Wednesday, 19 February 2014 to 7 August 2015.
Ngāi Tahu Research Consultation Committee
Te Komiti Rakahau ki Kāi Tahu

Nahaku noa, nā

Mark Brunton
Kawhikahuere Rangahau Māori
Research Manager Māori
Research Division
Te Whare Wānanga o Otago
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Email: mark.brunton@otago.ac.nz
Web: www.otago.ac.nz
Appendix 4(b)

(Pertinent section of SHDB Tikaka Best Practice Document)

Body parts, tissue and substances (removal, retention, return or disposal)

Regardless of how minor the body part, tissue or substance (e.g. nail clippings, hair, blood) is perceived to be, the Tikaka Best Practice – Removal of Body Parts policy will be followed. This includes whenua (placenta) and genetic material.

All discussions will be non-judgemental and follow an informed process.

Staff action:

Ensure the patient and whānau receive a full and clear explanation about the procedure and options for removal, retention, return or disposal as early as possible.

Support this by:

- Offering further support from appropriate staff (e.g. kaitakawaenga).
- Returning the body part, tissue or substance in a way that is consistent with tikaka and in consultation with appropriate staff.
- Recording and carrying out the wishes of the patient and whānau if the original purpose of retention changes.
- Following existing protocols to return unconsented body parts, tissue and substances.
- Giving a full explanation of how and where disposal or burial will be carried out if return or retention is not requested.
- Documenting all discussions and decisions in the clinical notes.
- Ensuring all body parts, tissue and substances are correctly labelled.

Organ and Tissue Donation

Refer to the Tikaka Best Practice – Organ and Tissue Donation policy.

Body parts, tissue and substances
The following table contains the spectroscopy readings obtained from the NanoDrop (DNA-50 function with NanoDrop 1000, v3.7.1, Thermo-Fisher Scientific) for the DNA extraction comparison experiment. The boiling, Chelex and Phenol-Chloroform readings were obtained from 1 mL (each) of O111:K88:H− culture, whereas 0.2 mL was processed with the PrepMan. The extracted samples were diluted (based on the average of the triplicate reads shown here) to account for this difference in volume before comparison of their relative amplification of the uidA gene by RT-PCR.

<table>
<thead>
<tr>
<th>Extraction method sample</th>
<th>NanoDrop readings (ng/µL)</th>
<th>260/280 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiling</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>614.5</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>594.6</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>604.4</td>
<td>1.97</td>
</tr>
<tr>
<td>Chelex</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>548.4</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>550.1</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>548.3</td>
<td>2.01</td>
</tr>
<tr>
<td>Phenol-Chloroform</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>576.1</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td>586.5</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td>593.7</td>
<td>2.04</td>
</tr>
<tr>
<td>PrepMan</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>235.9</td>
<td>2.02</td>
</tr>
<tr>
<td></td>
<td>208.7</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>212.8</td>
<td>2.11</td>
</tr>
</tbody>
</table>

The culture sample processed by boiling extraction appears to have the greatest DNA concentration, but this method was found to have the lowest yield in the subsequent PCR assay. Based on the concurrent gel photo (see Figure 19(a)), it appears that much of the DNA that was extracted by the boiling method was highly fragmented, and so it is likely the NanoDrop concentration shown is comprised of poor quality DNA. The 260/280 ratio indicates that the reading is not influenced by protein carryover from the extraction of the broth, although it is possible other particles in solution may have contributed to concentration bias. The lower concentration of PrepMan as extracted likely contributed to its faintness on the gel, although this method also showed significantly more fragmentation than that of the Chelex method.

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### Appendix 6

**stx-bearing *E. coli* genomes**

<table>
<thead>
<tr>
<th>NCBI <em>E. coli</em> strains (completed sequences)</th>
<th>Genome Size (Mb)</th>
<th>GC%</th>
<th>NCBI chromosome FASTA seq ref no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>O103:H2 str. 12009</td>
<td>5.52486</td>
<td>50.68</td>
<td>NC_013353.1</td>
</tr>
<tr>
<td>O111:H- str. 11128</td>
<td>5.76608</td>
<td>50.42</td>
<td>NC_013364.1</td>
</tr>
<tr>
<td>O157:H7 str. EC4115</td>
<td>5.70417</td>
<td>50.39</td>
<td>NC_011353.1</td>
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<tr>
<td>O157:H7 str. TW14359</td>
<td>5.62274</td>
<td>50.46</td>
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<tr>
<td>O26:H11 str. 11368</td>
<td>5.85553</td>
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<td>NC_013361.1</td>
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<tr>
<td>Xuzhou21</td>
<td>5.51674</td>
<td>50.38</td>
<td>NC_017906.1</td>
</tr>
<tr>
<td>O145:H28 str. RM13514</td>
<td>5.73729</td>
<td>50.67</td>
<td>NZ_CP006027.1</td>
</tr>
<tr>
<td>O145:H28 str. RM13516</td>
<td>5.55901</td>
<td>50.59</td>
<td>NZ_CP006262.1</td>
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<tr>
<td>O157:H7 str. SS17</td>
<td>5.65594</td>
<td>50.38</td>
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<td>O157:H7 str. EDL933</td>
<td>5.62052</td>
<td>50.35</td>
<td>AE005174.2</td>
</tr>
<tr>
<td>O157:H7 str. Sakai RIMD 0509952</td>
<td>5.59448</td>
<td>50.45</td>
<td>NC_002695.1</td>
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<tr>
<td>UMNF18</td>
<td>5.6353</td>
<td>50.58</td>
<td>NZ_AGTD01000001.1</td>
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<tr>
<td>O104:H4 str. 2011C-3493</td>
<td>5.43741</td>
<td>50.63</td>
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<tr>
<td>O104:H4 str. 2009EL-2050</td>
<td>5.43817</td>
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<tr>
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<td>NC_018661.1</td>
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<tr>
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<td>50.59</td>
<td>NZ_CP007133.1</td>
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<tr>
<td>O145:H28 str. RM12581</td>
<td>5.73729</td>
<td>50.67</td>
<td>NZ_CP007136.1</td>
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</tbody>
</table>

Mean values

<table>
<thead>
<tr>
<th>Genome Size (Mb)</th>
<th>GC%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.609073529</td>
<td>50.53</td>
</tr>
</tbody>
</table>
Appendix 7

Non stx-bearing *E. coli* genomes

<table>
<thead>
<tr>
<th>Non STEC strains</th>
<th>Genome Size (Mb)</th>
<th>GC%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> str. K-12 substr. MG1655</td>
<td>4.64165</td>
<td>50.8</td>
</tr>
<tr>
<td><em>Escherichia coli</em> CFT073</td>
<td>5.23143</td>
<td>50.5</td>
</tr>
<tr>
<td><em>Escherichia coli</em> BL21(DE3)</td>
<td>4.55895</td>
<td>50.8</td>
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<tr>
<td><em>Escherichia coli</em> SE11</td>
<td>5.15563</td>
<td>50.75</td>
</tr>
<tr>
<td><em>Escherichia coli</em> SE15</td>
<td>4.83968</td>
<td>50.71</td>
</tr>
<tr>
<td><em>Escherichia coli</em> UTI89</td>
<td>5.17997</td>
<td>50.61</td>
</tr>
<tr>
<td><em>Escherichia coli</em> 536</td>
<td>4.93892</td>
<td>50.5</td>
</tr>
<tr>
<td><em>Escherichia coli</em> APEC O1</td>
<td>5.49765</td>
<td>50.29</td>
</tr>
<tr>
<td><em>Escherichia coli</em> E24377A</td>
<td>5.24929</td>
<td>50.54</td>
</tr>
<tr>
<td><em>Escherichia coli</em> HS</td>
<td>4.64354</td>
<td>50.8</td>
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<td><em>Escherichia coli</em> B str. REL606</td>
<td>4.62981</td>
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<tr>
<td><em>Escherichia coli</em> ATCC 8739</td>
<td>4.74622</td>
<td>50.9</td>
</tr>
<tr>
<td><em>Escherichia coli</em> str. K-12 substr. DH10B</td>
<td>4.68614</td>
<td>50.8</td>
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<td><em>Escherichia coli</em> SMS-3-5</td>
<td>5.21538</td>
<td>50.5</td>
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<tr>
<td><em>Escherichia coli</em> BW2952</td>
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<tr>
<td><em>Escherichia coli</em> DH1</td>
<td>4.57094</td>
<td>50.8</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ’BL21-Gold(DE3)pLysS AG’</td>
<td>4.57094</td>
<td>50.8</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O55:H7 str. CB9615</td>
<td>5.45235</td>
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<tr>
<td><em>Escherichia coli</em> IHE3034</td>
<td>5.10838</td>
<td>50.7</td>
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<tr>
<td><em>Escherichia coli</em> 55989</td>
<td>5.15486</td>
<td>50.7</td>
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<tr>
<td><em>Escherichia coli</em> IAI1</td>
<td>4.70056</td>
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<td><em>Escherichia coli</em> S88</td>
<td>5.16612</td>
<td>50.66</td>
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<tr>
<td><em>Escherichia coli</em> O127:H6 str. E2348/69</td>
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<td><em>Escherichia coli</em> 042</td>
<td>5.35532</td>
<td>50.58</td>
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<td><em>Escherichia coli</em> KO11FL</td>
<td>5.02932</td>
<td>50.78</td>
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<td><em>Escherichia coli</em> ABU 83972</td>
<td>5.13296</td>
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<td><em>Escherichia coli</em> UM146</td>
<td>5.10756</td>
<td>50.61</td>
</tr>
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<td><em>Escherichia coli</em> W</td>
<td>5.00886</td>
<td>50.78</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ETEC H10407</td>
<td>5.32589</td>
<td>50.73</td>
</tr>
<tr>
<td>Mean values</td>
<td>4.98435</td>
<td>50.68</td>
</tr>
</tbody>
</table>
Appendix 8

Genome size and molecular weight calculations
(Example shown here is for str-bearing genome size, but the same process is used in other calculations)

Molecular weight (mol L⁻¹) of polymerised nucleotides

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Molecular Weight (mol L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosine</td>
<td>289.2</td>
</tr>
<tr>
<td>Guanine</td>
<td>329.2</td>
</tr>
<tr>
<td>Thymine</td>
<td>304.2</td>
</tr>
<tr>
<td>Adenine</td>
<td>313.2</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(TABLE 5) str⁺ Mean genome size 5.609073529 Mean genome GC% 50.53

Equation:

To attain molecular weight of genome

\[
\frac{(%GC \times \text{total length})}{100} \times 618.4 + \frac{(100 - \%GC \times \text{total length})}{100} \times 617.4
\]

Then

\[
\frac{\text{(molecular weight value)}}{6.022 \times 10^{23}}
\]

Where \(6.022 \times 10^{23}\) refers to Avogadro’s constant. With mean str⁺ values, this equates to an average molecular weight of 3.465 876 526g per \(6.022 \times 10^{23}\) genomes, or 5.755357898 femtograms per genome (dividing the weight by the constant). Dividing the measured weight of extracted DNA by this amount in the same units provides the number of genomes contained in the sample by weight (for example 1 ng of extracted is expected to contain \(173,751\) genomes, this estimate is rounded to the nearest thousand to account for pipetting error). This number was then applied to a standard curve using ViiA7 or 7500 software and could be used to quantify numbers of genomes based on Ct values on qPCR plates that had an included DNA standard.