Genomically Informed Custom Selective Enrichment of Shiga-toxigenic *E. coli* (STEC) outbreak strains in foods

by

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Abstract

Foodborne bacterial outbreaks caused by Shiga-toxigenic *E. coli* (STEC) continue to place a burden on public health systems in developed countries. The STEC family of pathogens is biochemically diverse and current microbiological methods for detecting STEC may be encumbered by lack of a universal selective enrichment method, especially against high levels of background microbiota. A method has been previously described where genomic antimicrobial resistance (AMR) prediction tools are used to inform selection of a custom enrichment technique for recovery of a target STEC strain from ground beef. Here we build upon that concept and demonstrate the broader applicability of custom selective enrichment using recovery of five unique STEC strains from ground beef, bean sprouts and spinach. Drastically improved recovery of STEC strains from microbiologically diverse foods was shown for all 9 antibiotics examined in this study. The ability to accurately leverage AMR traits in specific pathogens for their recovery from high levels of background microbiota suggests this approach can be universally applicable in support of foodborne illness investigations.

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List of Abbreviations

µg: Microgram

AMR: Antimicrobial Resistance

ARG: Antimicrobial resistance gene

ARGD: Antibiotic Resistance Gene Database

BAM: Bacteriological Analytical Manual

BD: Bloody diarrhea

BHI: Brain Heart Infusion

BLAST: Basic Local Alignment Search Tool

CFIA: Canadian Food Inspection Agency

CFU: Colony forming unit

CHL: Chloramphenicol

DNA: Deoxyribonucleic acid

eae: Intimin gene

EHEC: Enterohemorrhagic *Escherichia coli*

FDA: Federal Drug Administration

GEN: Gentamicin

HUS: Haemolytic Uremic Syndrome

LEE: Locus of enterocyte effacement

MFLP: Laboratory Procedures for the Microbiological Analysis of Foods

mg: Milligram

MIC: Minimum Inhibitory Concentration

mL: Milliliter

mTSB: modified Tryptone Soya Broth

NCBI: National Center for Biotechnology Information

OLC: Ottawa Laboratory Carling

PCR: Polymerase Chain Reaction

RAJ: Recto-Anal Junction

ROS: Radical Oxygen Species

SNP: Single Nucleotide Polymorphism

STEC: Shiga Toxigenic *Escherichia coli*

STE-PCR: Shiga toxin and *eae* Polymerase Chain Reaction

stx: Shiga Toxin gene

ter: Tellurite associated gene

TMP: Trimethoprim

VT-PCR: verotoxin Polymerase Chain Reaction

WGS: Whole Genome Sequencing

Chapter 1: Introduction

1.1 Foodborne Disease and Outbreak Response

Foodborne disease is cause for major public health issues across the globe. In Canada there an estimated 4 million cases of foodborne illness each year related to 30 known pathogens (Thomas et al., 2013). Of the 4 million cases per year in Canada, 33,000 of those are related to the ingestion of Shiga-toxin producing *Escherichia coli* (STEC)(Thomas et al., 2013). The terms "Verotoxin-producing *E. coli*" (VTEC) and "enterohemorrhagic *E. coli*" (EHEC) are used synonymously with STEC. Investigation of foodborne illness outbreaks relies on collaboration between public health laboratories and food inspection professionals to identify and remove contamination sources. Un-timely removal of contaminated food lots can place a significant burden on the healthcare system, which makes it crucial for food testing protocols to link the case at hand with salient characteristics of bacterial isolates within the shortest time frame possible.

A foodborne outbreak is an event in which several people have developed a foodborne illness with similar symptoms from the same contaminated food. Public health surveillance programs such as the Canadian Notifiable Disease Surveillance System (Totten et al., 2019), National Enteric Surveillance System (Nesbitt et al., 2012), FoodNet Canada (Public Health Agency of Canada, 2019), PulseNet (Nadon et al., 2017) and others are used to monitor and detect clusters of similar health events. In order to be captured by a surveillance program, a person must seek medical care, submit a clinical sample for testing, the test has to be performed successfully by a private laboratory or hospital and then a report sent to the appropriate provincial/national levels. In cases where testing resources aren't available, surveillance can be performed through different

measures such as absences from schools and illnesses after an event or within a closed environment (e.g., long term care home) (Blais et al., 2019a). This type of surveillance can identify outbreaks and clusters of disease, but it does not provide information on trends in illness occurring outside of these groups, nor can it identify a causative organism (Blais et al., 2019a).

In recent years, Whole-Genome sequencing (WGS) has provided many advances in foodborne outbreak surveillance with its ability to provide the DNA fingerprint of an organism in both an accurate and timely manner. WGS based methods of surveillance has been implemented in foodborne monitoring and detection programs in many countries and has been conducted on foodborne pathogens recovered by the CFIA since 2014 (Cooper et al., 2020). Due to its timely generation of accurate and reliable results, clinical isolates from sick patients are often subjected to WGS analyses to determine the causative organism for an enteric illness.

Interpretation of WGS output data is difficult and requires extensive bioinformatic knowledge, however, publicly available bioinformatics applications have been developed which provide accessible user-friendly tools for WGS analysis. High-resolution strain discrimination can be achieved by two methods of post-sequencing analysis: (1) Single Nucleotide Variant (SNV) analysis, where the isolates are compared to a closely related reference, and (2) multilocus sequence typing (MLST), which works by assessing sequence variations in the coding region of genes. SNV analysis is more discriminatory because of its ability to assess variations in noncoding regions (Brown et al., 2019). This method is preferentially used by the FDA because it is deemed more fit to identify products associated with an outbreak (Timme et al., 2018). On the other hand, MLST is

more computationally available and less bioinformatically demanding (Brown et al., 2019).

In addition to the Canadian Food Inspection Agency, who have produced their own set of custom tools for WGS analysis (Carrillo et al., 2016; Lambert et al., 2015), the Center for Genomic Epidemiology (CGE) has developed over 30 web-based tools for analysis of bacterial WGS. This has enabled identification of acquired antibiotic resistance genes in genomes of interest using CGE tools such as ResFinder. A database of acquired antibiotic resistance genes, which is the foundation of ResFinder, is compiled from existing databases, e.g., the antibiotic resistance genes database (ARDB). New genes are continuously being added as they are described in the literature, currently there are over 2000 resistance genes covering 14 types of antimicrobial resistance agents within the databases used by ResFinder (Bortolaia et al., 2020). Using raw reads or assembled sequences as inputs, ResFinder searches the databases for matching resistance genes from the input genome fragments using a Kmer-based mapping first, followed by use of a basic local alignment search tool (BLAST) for identifying matching sequences (Bortolaia et al., 2020). BLAST identifies homologous sequences using a heuristic method which initially finds short matches between two sequences. After initial match, BLAST attempts to start local alignments from these initial matches (Ye et al., 2006). ResFinder does not have the ability to identify point mutations, however, other CGE tools exist to do so, such as MINTyper (Bortolaia et al., 2020). Through use of these publicly accessible bioinformatics tools, complex WGS data can be refined to a useable form for health care and food inspection professionals.

1.2 STEC

E. coli are a harmless commensal gut microorganism, but some variants which have acquired virulence factors (*stx1, stx2*) are pathogens capable of causing a wide range of symptoms in a human host. STEC are a closely related form of *E. coli* differentiated by the presence of at least one or more Shiga toxins (Karmali et al., 1983, 1985).

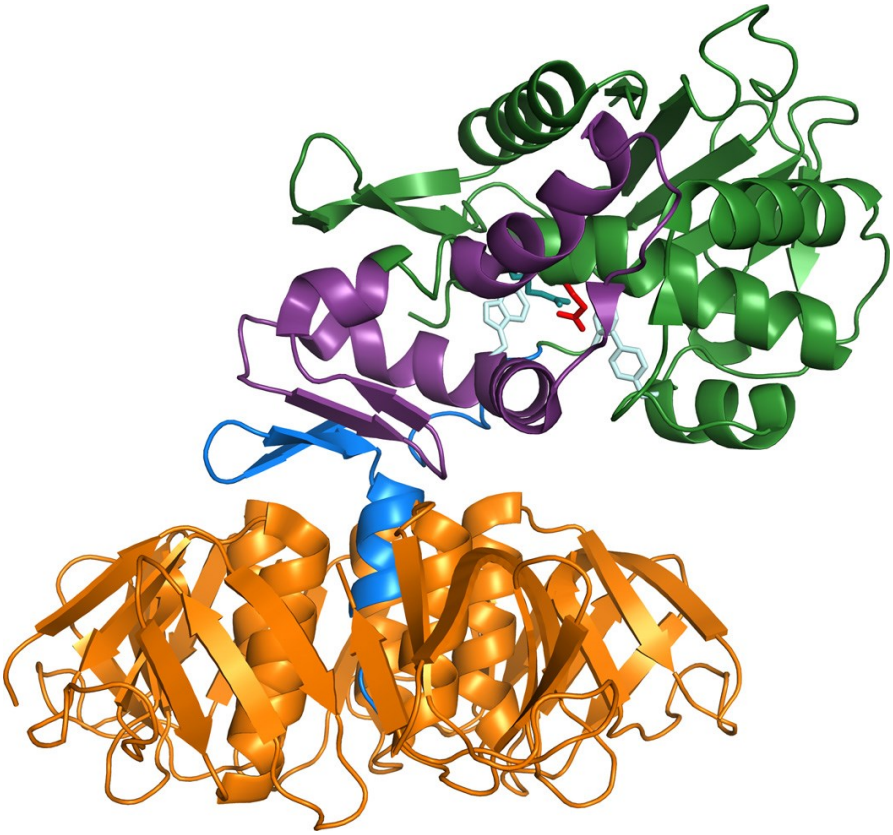

Figure 1: Ribbon diagram of Stx1 crystal structure.[1]

Shiga toxins are bacterial protein toxins of the AB5 family and homologues of the Shiga toxin for *Shigella dysenteriae* (Melton-Celsa, 2014). There are two main classes of

[1] The B pentamer is shown in orange and the A2 in blue. The majority of the A1 is depicted in green except for the region that interacts with the ribosome, which is shown in purple. The active residue 167 is red and other active-site side chains are pale blue. The A2 chain is medium blue and the B subunits are orange. The structure (1R4Q) was drawn with PyMOL Molecular Graphics System, Version 1.5.0 Schrödinger, LLC.

Shiga toxins; *stx1* and *stx2.* Production of Shiga toxins are encoded by closely related bacteriophages (Donnenberg & Whittam, 2001). Each Shiga toxin is comprised of a single A subunit and a pentamer of identical B subunits. The B subunit is responsible for binding to host cells, and the A subunit is taken up by the cell and targets the 28s rRNA of the 60S ribosome subunit, which becomes depurinated at a specific adenosine residue, terminating protein synthesis leading to cell death (Baranzoni et al., 2016). Shiga toxin production is dependent on induction of the *stx* phage (Melton-Celsa, 2014).There are three subtypes of *stx1* (a, c, d) and seven *stx2* subtypes (a, b, c, d, e, f, g). Difference in pathogenicity between subtypes has been observed, where *stx2* is more likely to cause severe illness than *stx1* (Baranzoni et al., 2016; Capps et al., 2021).

Infection from STEC can give a range of symptoms including no noticeable illness, bloody diarrhea (BD), and in severe cases progresses to hemolytic uremic syndrome (HUS). HUS occurs as a result of the Shiga toxin passing from the digestive tract into the bloodstream and can create a triad of symptoms; acute renal failure, thrombocytopenia and haemolytic anemia (Welinder-Olsson & Kaijser, 2005). HUS is a potentially fatal complication and develops in up to 22% of individuals with an enteric illness (Valilis et al., 2018). Children and the elderly are at increased risk of developing severe complications from an STEC infection.

STEC typically require additional virulence factors other than Shiga toxins to cause infection. Presence of additional toxin encoding genes such as subtilase (s*ubA*) can increase virulence in strains, as well as the presence of hemolysins (*ChuA, HylA*) which can influence iron acquisition and virulence of STEC (Bosilevac & Koohmaraie, 2011). One of the most recognized virulence factors contributing to a pathogenic STEC are

those associated with adherence. STEC that contain an adherence factor can attach to host cells and are classified as a subset of STEC called Enterohemorrhagic *E. coli* (EHEC). The intimin protein, encoded by the *eae* gene, is located in the locus of enterocyte effacement (LEE) and is responsible for creating attachment and effacing lesions on the host cell, which allows attachment to the host, a crucial step in the pathogenesis of STEC. *AggR*, a transcriptional activator of adherence, also facilitates increased pathogenicity (Bernier et al., 2002; Boisen et al., 2019; Nataro et al., 1994).

The main source of STEC are ruminant animals, specifically cattle, which can account for over half of human cases of STEC infection (Nguyen & Sperandio, 2012). Ruminants like cattle transmit STEC via fecal matter, where once shed into the environment they can remain viable for 7 weeks in a warm environment and up to 11 weeks in a cold environment (Wang, Zhao, & Doyle, 1996). STEC can then be acquired by humans through consuming contaminated bovine-related products such as meat and dairy (Nguyen & Sperandio, 2012). Ground beef becomes contaminated with STEC during the slaughtering process, where the intestinal contents of the cattle become mixed with the meat being harvested, effectively inoculating it with STEC. Feces and manure containing STEC can also contaminate water supplies by being washed off via rainfall. Not all ruminants are a reservoir for STEC, but unlike humans, STEC colonization in cattle is asymptomatic, and can be difficult to track. Cattle lack expression of Gb3 on the vascular endothelium, the presence of which promotes much of the pathology associated with STEC infections in humans (Pruimboom-Brees et al., 2000). As a result, Shiga toxin cannot be endocytosed and transported to other organs to induce vascular damage in cattle. In contrast to humans where STEC colonizes in the colon, STEC colonizes the

recto-anal junction (RAJ) of cattle which is immune to the effects of Shiga toxins (Naylor et al., 2003).

1.3 Current Microbiological Testing Methods

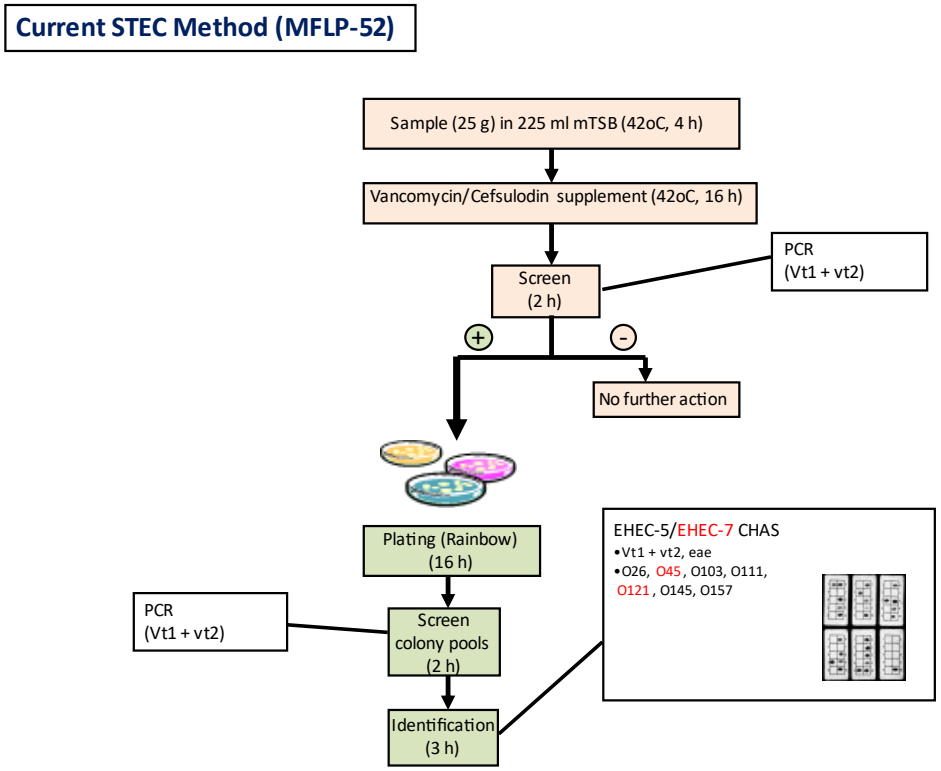

Figure 2: Workflow of the current method for Isolation and Identification of priority Shigatoxigenic *E. coli* in foods.[2]

Current methods for the detection of STEC from a food sample differ between various governing bodies and vary based on serotype. The general workflow used by these methods is consistent and are composed of Enrichment, Detection, Isolation and

[2] From Burton Blais, Section Head Research & Development, Canadian Food Inspection Agency, Ottawa Laboratory Carling.

Confirmation steps. Enrichment involves incubation of the food sample in broth media supplemented with various antimicrobial agents depending on the identified serotype. The enrichment increases growth of the target pathogen to detectable levels, while simultaneously limiting growth of any background microbiota. This step is crucial given that STEC may be present in extremely low and undetectable numbers in a food sample, while still causing illness. The Canadian Food Inspection agency uses a Vancomycin/Cefsulodin supplement (figure 2) to suppress gram positive bacteria (Blais et al., 2014), whereas the current U.S. method uses a Potassium tellurite/Cefixime supplement (Feng et al., 2020). Detection of STEC is determined by the presence of biomarkers in the enrichment broth specific for STEC (commonly *stx1* and *stx2*). If no biomarkers are detected in the enrichment, then the sample is considered negative; if biomarkers are detected for the pathogen, then the sample is considered a presumptive positive. The Canadian Food Inspection agency uses a VT-PCR assay developed in house (Blais et al., 2014) and the FDA uses a real-time PCR assay (Feng et al., 2020). Isolation of the pathogen from presumptive positive enrichment broths occurs via use of selective/differential agar media to recover a viable pathogenic cell. Both U.S. and Canadian food regulatory agencies use the highly differential Rainbow Agar O157 for this step (Blais et al., 2014, Feng et al., 2020). Biochemical characterization of isolated colonies provides confirmation of STEC recovery when the appropriate pattern of *stx1, stx2* and *eae* genes are present.

1.4 Issues recovering STEC from contaminated foods

STEC are a broad heterogenous group of bacteria that contain no unique physiological characteristics to form the basis of a selective or differential media

universal to all STEC. There are over 120 serotypes of confirmed STEC isolates, each with the potential to cause human illness (Mathusa et al., 2010). In the US and Canada, STEC O157:H7 are the most prominent in outbreaks, accounting for up to 50% of cases annually and have a higher association with HUS and other severe outcomes in patients (CDC FoodNet, 2020)(Bayliss et al., 2016). The prevalence of illness caused by non-O157 serotypes is likely underestimated due to the absence of routine testing (Thompson et al., 2005). For the recovery of O157:H7 STEC, resistance to certain antimicrobials (Tellurite, Cefixime and Novobiocin) are commonly leveraged to increase selectivity in the food testing programme (Boer & Heuvelink, 2000; Onoue et al., 1999; van Duynhoven et al., 2002). Novobiocin is an aminocoumarin antibiotic that inhibits bacterial DNA synthesis by targeting DNA gyrase and related DNA topoisomerase IV, leading to cell death (Smith & Davis, 1967). Tellurite is a rare mineral often used as potassium tellurite. The mechanism of action for potassium tellurite is not well understood, however, it is believed that tellurite exerts its toxicity through intracellular generation of reactive oxygen species, as well as an unbalancing of the thiol:redox buffering system, which is responsible for removal of reactive oxygen species (van Loi et al., 2015). The sensitivity to these antimicrobial compounds are inherently variable for STEC strains outside of the O157:H7 serotype, many may share similar traits to O157, but this resistance phenotype has not yet been reliably predicted in non-O157 STEC (Gill et al., 2014).

Food commodities such as ground beef, bean sprouts, and leafy greens have been highly associated with STEC outbreaks (Buchholz et al., 2011; Sharapov et al., 2016). STEC thrive in warm, wet, and nutrient rich environments, of which the gastrointestinal

tract of animals provides perfect refuge. Bean sprouts are also grown in a dark, warm, humid environment that provides an excellent breeding ground for bacteria. These conditions also provide efficient proliferation capacity for native microbiota in the food products, resulting in high levels of background microbial growth. Leafy greens such as spinach can be contaminated through contact with human handlers, as well as through unintended use of STEC contaminated fertilizer or water. This additional microbial growth creates a competitive environment for the bacteria which can hamper recovery of STEC in a food outbreak scenario, especially when low levels of contamination are present. STEC have an infectious dose of less than 100 cells in humans, which can result in very low levels of contamination causing human illness (Todd et al., 2008). Current methods for detection of STEC may not be able to identify scarce levels of contamination in a microbiologically diverse food sample, and until identification of one pathogenic cell per analytical sample is achieved, the opportunity for false negative reports of contamination remains.

Increasing recognition of non-O157:H7 STEC causing illness has been combatted by defining additional serogroups associated with high levels of STEC infection. The US has identified 7 major STEC serogroups (O26, O45, O103, O111, O121, O145, O157) that are of public health concern and commonly found in contaminated foods (Gould et al., 2013), however, the potential for appearance of a pathogenic STEC not described within this group is increasing, highlighted by a large outbreak of STEC O104 in Germany (Frank et al., 2014). The lack of serotype-independent methods for the recovery of non-O157 STEC, particularly from microbiologically complex food matrices, can

produce false negative results in a food investigation scenario and lead to prolonged outbreak investigations and increased illness.

1.5 Experimental Aim

Improvements in Polymerase Chain Reaction (PCR) and Whole Genome Sequencing (WGS) based detection methods have resulted in superior sensitivity, specificity, and timely resolution of genomic analysis, all while incurring a significant reduction in cost (Angers-Loustau et al., 2018; Carrillo et al., 2019). A number of tools are available to accurately interpret this data, giving insight into valuable characteristics of an organism. These improvements have made it possible to generate, sequence, and annotate bacterial genomes within the time frame of a food safety event (Lambert et al., 2015). Clinical isolates from patients with an enteric illness are often subjected to WGS as part of current outbreak investigation routines, which allows for food inspection laboratories to rapidly acquire useful genomic data on the pathogenic organism. During a food safety event, the application of AMR prediction tools on clinical WGS data can be parsed to determine whether a suitable AMR gene is present to aid in the selective recovery of the pathogen. An enrichment broth containing the indicated antibiotic would be formulated to promote growth of the pathogen from a complex food sample matrix, allowing enhanced recovery within the time frame of current food testing protocols.

Proof of concept has been shown for this approach using the recovery of multiple STEC strains from ground beef, where resistance to trimethoprim (TMP), gentamicin (GEN) and chloramphenicol (CHL) was predicted. With addition of the indicated antibiotic to a modified tryptone soya broth (mTSB) enrichment, the approach was able to greatly increase recovery of low levels of the model organism (Blais et al., 2019b;

Knowles et al., 2016). In order to be implemented on a national scale, and given the variable nature of STEC, this approach must be proven able to recover any STEC serotype from any food commodity, with a diverse arsenal of validated AMR genes and corresponding antibiotics available for use.

Here we aim to firmly establish the applicability of the method by investigating recovery of 5 additional non-O157:H7 STEC strains from ground beef and two additional high-risk food commodities; spinach leaves and bean sprouts. 14 separate AMR genes will be used to predict resistance to 9 different antibiotics comprising 5 different antibiotic resistance classes. Genomic predictors for tellurite and novobiocin resistance in non-O157:H7 STEC will be examined on a panel of 12 STEC strains. Additionally, the composition of the background microbiota and its effect on pathogen recovery will be studied by identifying competitive species in the enrichment broth using a VITEK microbial identification system. It is expected that some of the native microbiota will possess AMR characteristics, thus the ability to select against any commonly occurring AMR traits will be beneficial to the method. A comparative analysis of AMR genes in the background microbiota of foods, with the genome of a target pathogen in said food, will be used to select an AMR trait in the target pathogen that is least abundant in the background microbiota, thereby limiting non-target growth. The feasibility of a selective enrichment approach for detection of STEC from microbiologically complex food matrices, informed by comparative antimicrobial resistance trait rarity analysis is demonstrated.

Chapter 2: Materials & Methods

2.1 Chemicals and Reagents

Antimicrobials were obtained from Sigma-Aldrich (St. Louis, MO) and included chloramphenicol (C1919), florfenicol (F1427), gentamicin (G1264), kanamycin A (K1377), neomycin (N0401000), novobiocin (N6160), penicillin G (P3032), potassium tellurite (P0677) streptomycin (S6501), sulfadiazine (S8626), trimethoprim (T7883)

2.2 Bacterial Strains

Five STEC strains from the Ottawa Laboratory Carling (OLC) Canadian Food Inspection Agency culture collection (table 1) were selected as model target strains for recovery studies based on the presence of AMR genes (genomes previously sequenced and deposited at DDBJ/EMBL/GenBank under BioProject PRJNA319494).
Twelve additional STEC strains were selected for antimicrobial MIC testing (table 7). This panel was chosen from previously sequenced STEC strains in the CFIA OLC bacterial culture collection. Two O157 STEC were chosen on the expectation they will contain identifiable tellurite resistance genes; the remaining strains were all non-O157 STEC for which tellurite resistance was expected to be variable. Bacteria were routinely grown on Brain Heart Infusion (BHI) agar (Oxoid, Basingstoke, England) for 16 to 20 h at 37°C. Sequence data locations for all bacterial strains used in this study are located in table 2.

Table 1: Non-O157:H7 STEC strains obtained from CFIA's Ottawa Laboratory (Carling) Bacterial Culture Collection, used to replicate real-world contamination events of non-O157:H7 STEC in foods. [3]

Strain	Serotype	Virulence profile	Antimicrobial Resistance Genes
455	O111:H11	*stx1a, eae*	aph(3')-Ia, aph(6)-Id, sul1, tet(A), catA1, mdf(A), dfrA1
714	O111:H8	*stx1a, eae*	aph(3')-Ia, aph(6)-Id, aph(3")-Ib, aac(3), aadA5, sul1, sul2, tet(A), catA1, mdf(A), dfrA17, blaTEM
792	O121:H19	*stx2a, eae*	aph(6)-Id, aph(3")-Ib, aadA2, sul1, sul2, tet(A), floR, mdf(A), dfrA12, blaCMY
969	O103:H2	*stx2a, eae*	aph(6)-Id, aph(3")-Ib, sul2, tet(A), mdf(A), dfrA8, fosA7, blaTEM
1337	O2:H25	*stx2c, eae*	aph(6)-Id, aph(3")-Ib, sul2, tet(A), floR, mdf(A)

[3] Serotype and virulence profile identified using V-typer (Carrillo et al., 2016). Antimicrobial resistance genes identified using Resfinder (Bortolaia et al., 2020).

Table 2: **Sequence data location for all strains used in study**[4]

Strain	NCBI Accession or Biosample number
455	GCA_002531215.1
714	GCA_002530935.1
792	N/A
803	SAMN13333399
969	N/A
1337	SAMN13333971
1358	SAMN13333985
2243	SAMN10746426
2364	SAMN13334731
2383	SAMN11030305
2524	SAMN13334749
2526	SAMN11030313
3374	SAMN11373501
3407	SAMN13334875
3456	SAMN13334872
3483	N/A
3504	N/A

2.3 Antimicrobial Predictions

ResFinder Web server version 3.2 (http://www.genomicepidemiology.org/) was used to analyze STEC genomes (previously deposited in GenBank under BioProject PRJNA319494) for antibiotic resistance. Criteria for all ResFinder predictions were minimum 98% sequence ID and 60% coverage, as suggested by Zankari et al. (2013). Tellurite (*terA, terB, terC, terD, terE, terF, terZ*) and novobiocin (*gyrA, gyrB, parE*)

[4] All strains were obtained from the Canadian Food Inspection Agencies Bacterial Culture Collection at the Ottawa Carling Laboratory. Deposited aaccession numbers have the prefix "GCA", biosample numbers have the prefix "SAMN". For strains listed as N/A, sequence data has not yet been deposited. To access these strains, contact Dr. Catherine Carrillo, Research Scientist, Canadian Food Inspection Agency, Catherine.Carrillo@canada.ca / Tel: 613-759-1278.

related resistance genes were analyzed using a Basic Local Alignment Search Tool (BLAST) script (https://olc-bioinformatics.github.io/GeneSeekr).

2.4 Selective Recovery

Fresh food samples (ground beef, spinach, bean sprouts) were obtained from local retailers. To determine the microbial level within these foods, total aerobic counts were obtained by stomaching 25 g of sample in 225 mL of 0.1% Buffered Peptone Water (Oxoid Ltd.) and immediate plating of serial dilutions on Petrifilm aerobic count plates (Sigma-Aldrich Co.). 1 mL from each sample was incubated in triplicate for 16-20 h at 37°C. For recovery studies, 25 g of food sample was inoculated with a pre-determined amount of STEC and stomached in 225 mL of mTSB containing 10µg/mL or 100µg/mL of antibiotic (table 2). Antibiotic concentrations were chosen as decimal figures (i.e. 10 or 100 ug/mL) based on nearest breakpoint data (CLSI, 2018), which was done in recognition that standard MIC's are recorded in extremely controlled and precise growth environments which does not apply to our food sample matrix.

Table 3: Antibiotic dilution chart for selective recovery of STEC. [5]

Antibiotic Stock Concentration (mg/mL)	Volume of Antibiotic Stock Solution (mL)	Final Enrichment Concentration (µg/mL)
10	0.225	10
10	2.250	100

Enrichment cultures were incubated for 16 to 20 h at 37°C and screened for presence of STEC using STE PCR (Huszczynski et al., 2013). Diluted enrichment cultures were plated on MacConkey agar (Oxoid Ltd.) to limit variation in recovered colony morphology, which could influence random picking of colonies. Agar plates were

[5] Final enrichment concentrations used for each individual antibiotic can be found in table 4.

incubated overnight at 37°C to obtain isolated colonies (minimum 30 colonies per plate). All isolated colonies were individually added to 100 µL 1% (v/v) Triton X-100 (Oxoid Ltd.) and lysed at 100°C for 10 minutes. After cooling, the resulting lysates were confirmed using STE PCR. STE PCR targets *eae* (384-bp), *stx1* (182-bp) and *stx2* (200-bp) genes, where 5 µL of lysate was added to 45 µL of PCR master mix containing 2.5 U HotStar Taq, 1X HotStar PCR buffer (giving a final MgCl2 concentration of 1.5 mM; Qiagen Inc., Mississauga, Ontario, Canada), 200 mM (each) dNTP, and 0.2 mM each of the forward and reverse primers for *stx1* and *stx2*, 0.3 mM for *eae*, and 0.1 mM for the internal amplification control (IAC). The PCR was carried out in a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) using the following conditions: an initial denaturation cycle at 94°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and primer extension at 72°C for 1 min 30 s, with an additional 2 min at 72°C following the last cycle. Visualization of PCR bands was performed using a QIAxcel automated capillary electrophoresis device (Qiagen Inc.). Samples producing a band corresponding to at least one *stx* feature plus *eae* (ascertained by reference to a molecular-size ladder) was considered positive for STEC.

The recovery efficiency was calculated by determining the proportion of total colonies assayed that were positive for the expected patterns of *eae*, *stx1* and *stx2* amplicons for the inoculated strain. For each food commodity, an uninoculated sample was run in parallel as a control to determine that no STEC was present in the sample prior to inoculation.

2.5 VITEK analysis

The VITEK system (bioMérieux Inc., Montreal, Quebec, Canada) is used for microbial identification and antibiotic susceptibility testing, generating rapid and reliable results using individual bacterial colonies. The results VITEK generates are a direct result of phenotypic resistance to antimicrobials, and thus was chosen to reliably identify competing bacteria in our enrichment broths that persist as a result of antimicrobial resistance. Microbial identification using VITEK was performed on select non-STEC colonies which had grown under custom enrichment conditions. A total of 51 colonies were screened based on the enrichment treatment they received; 10 colonies chosen from ground beef sample enriched without antibiotic, 7 colonies from spinach enriched with Trimethoprim (TMP), 7 colonies from spinach enriched with Chloramphenicol (CHL), 7 colonies from spinach enriched with Gentamicin (GEN), 7 colonies from ground beef enriched with TMP, 7 colonies from ground beef enriched with GEN, and 7 colonies from ground beef enriched with CHL. Colony morphology consisted of either light pink or red circular colonies. All colonies were identified using the VITEK 2 GN ID card, which is the recommended method for identification of gram-negative bacteria using VITEK (Ling et al., 2001; Pincus, 2010).

2.6 Broth Microdilution for determination of Minimum Inhibitory Concentration of Potassium tellurite and Novobiocin in non-O157 STEC

Antimicrobial stock solutions of potassium tellurite (Sigma-Aldrich) and novobiocin (Sigma-Aldrich) were prepared at a concentration of 10mg/mL in deionized water and were filter sterilized through 0.22 μm filters. Doubling dilutions of

antimicrobials were performed in mTSB (Oxoid Ltd.) from 200 to 1.5 µg/mL. A panel of 12 STEC (table 6) grown individually in mTSB (Oxoid Ltd.) were inoculated (ca. 100 CFU) into 3 mL of antibiotic/broth solution and were incubated for 16 to 20 h at 37°C. Growth was assessed by measuring turbidity (A600) using a DensiCHECK Plus instrument (bioMérieux Inc., Montreal, Quebec, Canada) and was recorded using optical density.

Chapter 3: Results & Discussion

Recovery of STEC from foods can be extremely challenging due to complications culturing pathogens to detectable levels relative to background organisms present in different foods. The purpose of this study was to further develop and catalogue a WGS informed custom enrichment approach for the recovery of outbreak related STEC from foods. A food outbreak scenario was artificially simulated where a modified version of current STEC recovery methods is used to facilitate the recovery of pathogenic STEC when present at low levels in microbiologically complex food samples, where recovery of the pathogen is shown to be unlikely otherwise. These experiments build on the success of a previous study published by Blais and colleagues (Blais et al., 2019b).

3.1 Determining prevalence of Antibiotic Resistance in food bacteria

The driving force behind custom selective enrichment is the ability to reliably predict phenotypic antimicrobial resistance in a target organism. If no antimicrobial resistance is predicted for a target, then the method becomes ineffective. Additionally, if the background microbiota we are trying to suppress has high prevalence of a specific

antimicrobial resistance trait, that too can limit the effectiveness of the method if the indicated antimicrobial is used. A publicly available genomic antimicrobial resistance prediction tool (ResFinder), in combination with the National Centre for Biotechnology Information (NCBI) Pathogen Detection database, was used to determine prevalence of clinically relevant antibiotic resistance among *E. coli*, STEC, and other food commensal bacteria. There is limited data available for outbreak related STEC strains, thus the extremely comprehensive NCBI *E. coli* database was also used as an indicator for STEC AMR, given their close phylogenetic and biochemical relationship.

Predicted antimicrobial resistance for 21605 environmental *E. coli* isolates and 49480 clinical *E. coli* isolates from the NCBI database are shown in figure 2. Clinical isolates had slightly lower prevalence of antimicrobial resistance than environmental isolates, however, both categories followed a similar trend. Of 11 tested antimicrobial classes, resistance was predicted in *E. coli* to all but one; vancomycin resistance was not reported in either clinical or environmental isolates. Resistance to aminoglycoside and tetracycline antibiotic classes was the most reported at 40% of environmental isolates. Resistance to Colistin, Fosfomycin and MLS classes of antimicrobials was minimal at 5%.

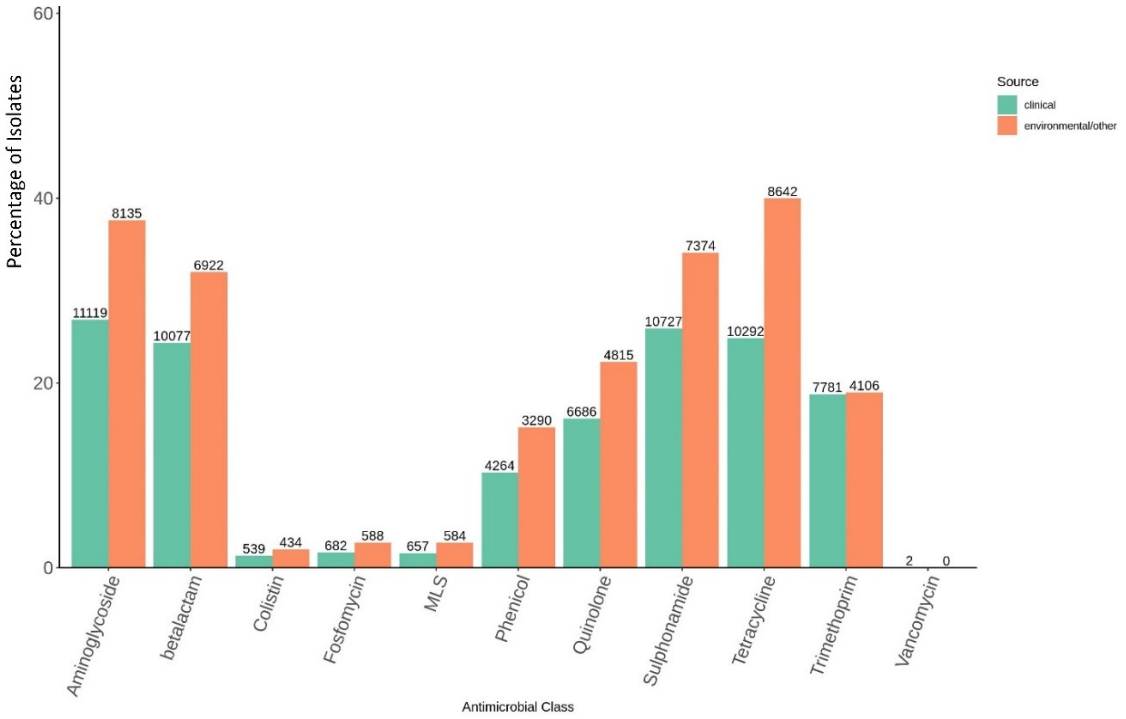

Figure 3: Prevalence of antimicrobial resistance genes by class for clinically and environmentally sourced *E. coli* from NCBI database. Bars indicate percentage of *E. coli* isolates carrying at least one resistance gene to the indicated antibiotic. Values at the top of bars indicate the number of isolates contained within that data point.

Genomic data for 298 STEC strains previously implicated in food outbreak events was obtained from the Canadian Food Inspection Agency's Ottawa Laboratory (Carling) Bacterial culture collection. Figure 4 represents predicted antimicrobial resistance in STEC obtained from various sources for non-O157 and O157:H7 STEC. Aminoglycoside, sulphonamide and tetracycline resistance was most abundant in STEC which follows the same trend as the much larger *E. coli* dataset. STEC isolates obtained from veal were the most likely to contain an instance of antimicrobial resistance. Lamb derived isolates were the least likely to carry an acquired resistance trait.

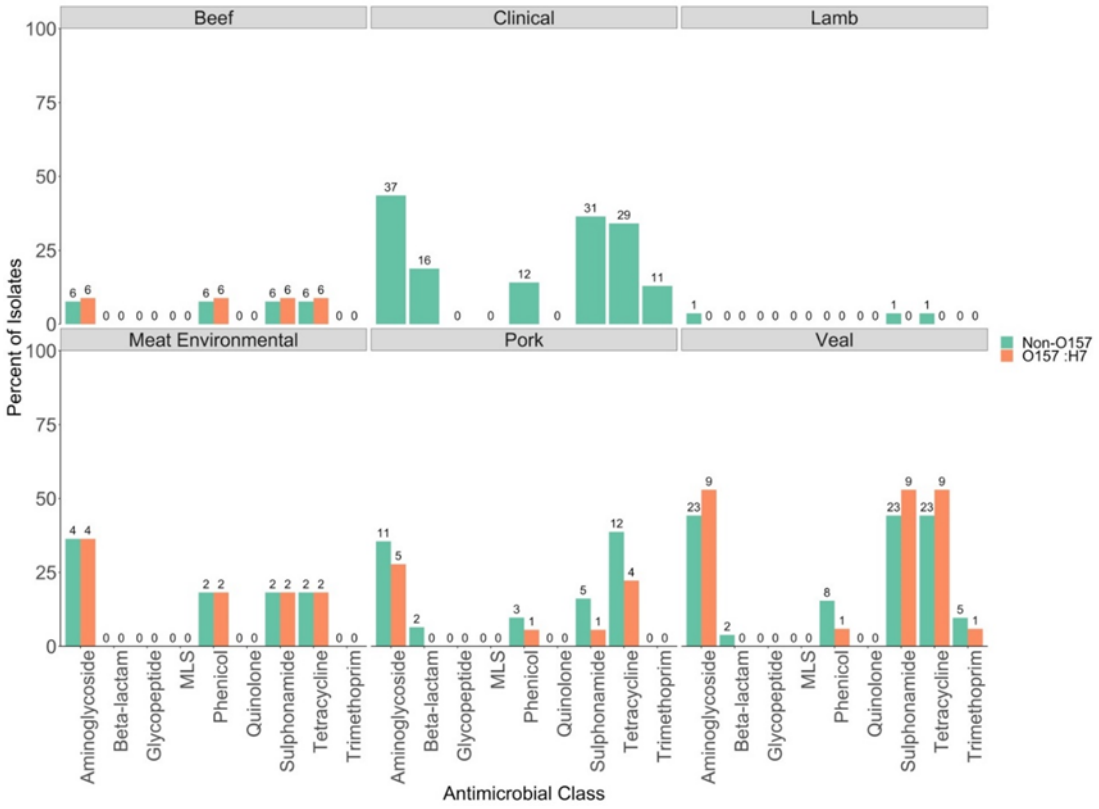

Figure 4: Prevalence of ARG's in STEC previously implicated in food safety events, obtained from the Canadian Food Inspection Agencies Ottawa Laboratory (Carling) Bacterial Culture Collection. Bars indicate percentage of STEC isolates carrying at least one resistance gene to the indicated antibiotic. Values at the top of bars indicate the number of isolates contained within that data point.

E. coli are intrinsically susceptible to many clinically relevant antimicrobials, but this bacterial species has great capacity to accumulate external resistance genes (Johnson & Russo, 2005). The result is a diverse range of antimicrobial resistances across all *E. coli* and related species. Over 40% of *E. coli* isolates on the NCBI database are resistant to at least one clinically relevant antibiotic agent as predicted by ResFinder (figure 3). Prevalence of AMR in environmental isolates was greater than in clinical isolates for all examined antibiotic classes (figure 3). One potential reason for this trend is the overuse of antimicrobials in raising farm animals, which are ultimately consumed by humans. The FDA reported in 2018 that of all antimicrobials sold in the United States that were used in animal agriculture, 52% are also used clinically in humans (United States, 2019). This

creates a profound driving force for the emergence of antimicrobial resistant bacteria, which can ultimately be transferred to human microbiomes. Clinical bacterial resistance could be the result of excess use of antibiotics in humans, but also through infections acquired from environmental sources where antibiotics are used.

STEC were shown to follow a less prevalent but similar trend in acquired resistance as *E. coli*, which can be expected from such closely related species (figure 3,4). 24% of all sequenced STEC in the CFIA OLC's bacterial culture collection, all of which had been directly isolated from previous contaminated food lots, were resistant to at least one antibiotic, compared to 40% identified in *E. coli* from the NCBI database. However, when comparing only clinical isolates from the CFIA the prevalence is much higher, where 37% of clinical isolates had aminoglycoside resistance, 31% had sulphonamide resistance, and 29% had tetracycline resistance (figure 4). Given our approach begins with the analysis of clinical outbreak strains, the higher prevalence of AMR in clinical isolates bodes well for our approach. The CFIA OLC STEC collection and NCBI database are constantly updated and can be repeatedly analyzed to identify potential new AMR targets as they arise.

E. coli and its related species make up only a portion of the food microbiome. The NCBI Pathogen Detection project is a centralized system that integrates sequence data for bacterial pathogens obtained from food, the environment, and human patients. A number of public health agencies in Canada, the US and internationally are collecting samples from these sources to facilitate active, real-time surveillance of pathogens and foodborne disease. This makes NCBI's Pathogen detection database a useful tool for identifying potentially competitive genera of bacteria that may arise foods. Competition arises when

the background microbial community competes for resources (e.g., nutrients) and can hamper growth of STEC in the sample. Two main factors will drive competition in the background microbiota; the presence of AMR genes that will confer resistance to the chosen antimicrobial for selective enrichment, and the initial magnitude of commensal bacteria at the time of enrichment. Bacteria can also use a variety of mechanisms to prevent growth of competing organisms. Production of bacteriophages, bacteriocins and other antibiotics are all ways certain bacteria can achieve microbial antagonism (McMahon, 2018).

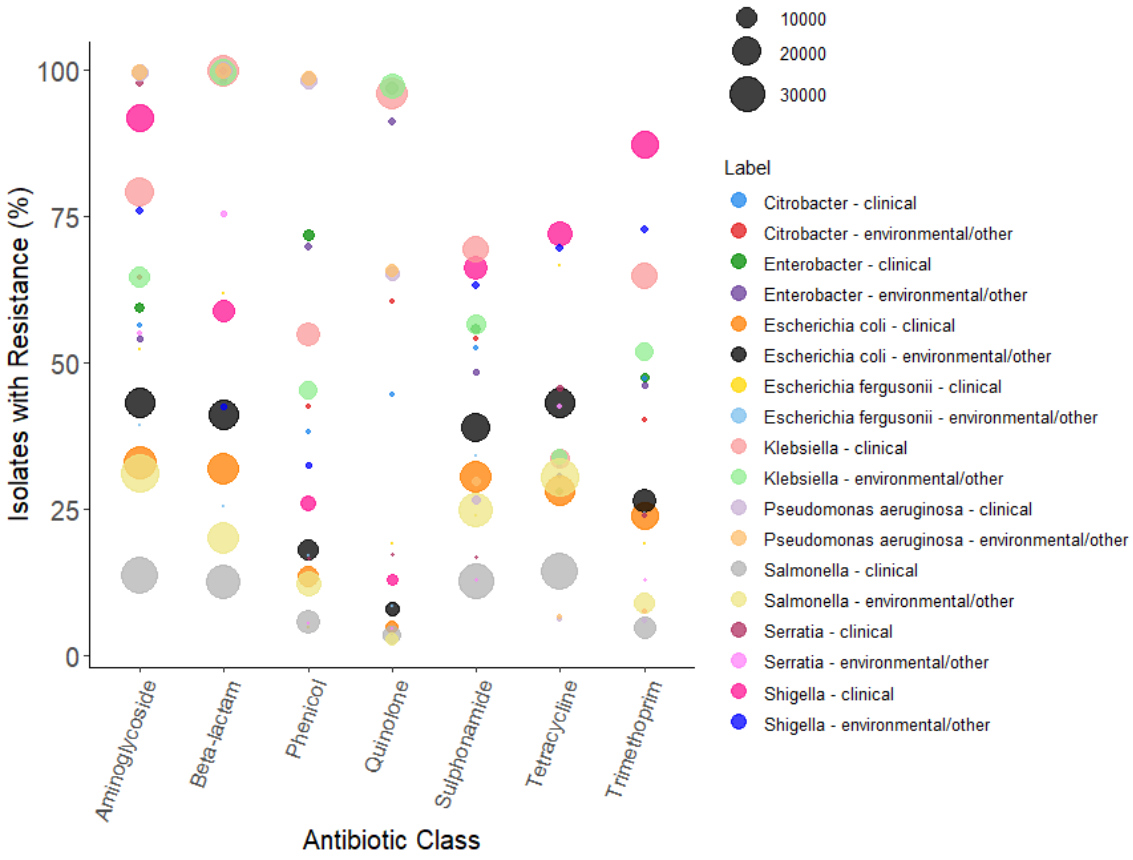

Figure 5: Prevalence of Antimicrobial resistance genes per genus of common genera found in foods. Circle diameter is indicative of sample size for the data point, where a larger circle indicates a larger sample size for that genus.[6]

Resistance to antibiotics was shown to be prevalent among almost all genera (figure 5). Resistance profiles were variable, 99% of environmental *Pseudomonas aeruginosa* species harbored antibiotic resistance genes for Aminoglycosides and/or Beta-lactams, but only 8% harbored resistance genes for tetracycline and trimethoprim. Trimethoprim resistance was present in 85% of clinical *Shigella*, but only represented 9% in clinical *Salmonella*. This highlights the diversity of commensal organisms that can be

[6] Data obtained on 2021-01-10 from NCBI's pathogen detection database at https://ftp.ncbi.nlm.nih.gov/pathogen/Results/. ggplot2 was used to curate information (Wickham, 2011).

present in a food sample, which can produce highly variable AMR characteristics that may vary on a case-by-case basis. Steps can be taken to select an antimicrobial for which resistance is least likely to occur natively in the sample. The application of a comparative antimicrobial trait rarity analysis could identify AMR traits predicted to be in the native sample, where any commonly occurring AMR traits in the sample would be selected against when choosing an antibiotic to isolate a target pathogen.

3.2 Reproducing sets of Antimicrobial Resistance Gene predictions and antibiotics for recovery of various STEC strains from new food types

STEC strain OLC-714 was selected as the main model organism for this study based on its genotype exhibiting multiple AMR profiles identified by ResFinder analysis. Three antibiotics from different antibiotic classes were chosen as models for selective enrichment based on differing resistance mechanisms; aminoglycosides (Gentamicin, GEN), diaminopyrimidines (Trimethoprim, TMP), and phenicols (Chloramphenicol, CHL). Fresh store-bought food samples (ground beef, spinach, bean sprouts) inoculated with different concentrations of STEC model strain were enriched in mTSB containing one of the antibiotics for which resistance was predicted. The antibiotic concentration for TMP, GEN and CHL were selected based on the success of a previous study (Blais et al., 2019b). The choice of enrichment broth and agar media were selected based on current STEC recovery methods, omitting the addition of currently used antimicrobial agents and substituted with our own genomically-informed selection of antimicrobials. In this way, the proposed custom selective enrichment method will have little variance from the current technique. Ground beef, spinach leaves and bean sprouts were selected as they are

commonly implicated in STEC events and are known to contain high levels of background microbiota (Pires et al., 2019). This will help increase the competitiveness of the enrichment environment, ensuring the method is tested under the most rigorous conditions. The level of background microbiota present in the food samples pre-enrichment was $2.25x10^6$ CFU/g, $5.95x10^7$ CFU/g and $2.26x10^4$ CFU/g for the ground beef, bean sprouts, and spinach respectively. Overnight enrichments devoid of antibiotic supplementation produced no recovery of the target strain on MacConkey agar for any of the tested food products at any inoculum level (table 3). At ~300 CFU inoculum, all tested antibiotics in all foods produced 100% recovery of the target strain. With ~30 CFU inoculum, 64% recovery of the target STEC from Spinach enriched with Chloramphenicol was achieved, all other recoveries at that inoculum were 99% or higher. Recovery of the target from 3 CFU inoculum was weaker in both spinach and bean sprout samples, with CHL and GEN supplementation producing no recovery in Spinach, and TMP no recovery in sprouts. Instances of zero target recovery could have been caused by lack of inoculation, where low-level sampling of the target pool may result in no inoculum being received due to the stochastic nature of cell distribution.

Table 4: Recovery of OLC714 STEC inoculated at multiple levels from ground beef, spinach and bean sprouts using a custom enrichment method. [7]

Food Commodity	Background microbiota (CFU/g)	Antibiotic	AMR gene	Inoculum (CFU)	Recovery (%)
Ground beef	$2.25x10^6$	None	N/A	3	0
				22	0
				240	0
		CHL (10 µg/mL)	*CAT*	3	52
				22	100
				240	100
		GEN (100µg/mL)	*aac(3)-IId*	3	100
				22	100
				240	100
		TMP (100µg/mL)	*dfrA17*	3	100
				22	100
				240	100
Bean Sprouts	$5.95x10^7$	None	N/A	6	0
				53	0
				352	0
		CHL (10 µg/mL)	*CAT*	6	20
				53	100
				352	100
		GEN (100µg/mL)	*aac(3)-IId*	6	65
				53	100
				352	100
		TMP (100µg/mL)	*dfrA17*	6	0
				53	99
				352	100
Spinach	$2.26x10^4$	None	N/A	3	0
				23	0
				246	0
		CHL (10 µg/mL)	*CAT*	3	0
				23	64
				246	100
		GEN (100µg/mL)	*aac(3)-IId*	3	0
				23	99
				246	100
		TMP (100µg/mL)	*dfrA17*	3	88
				23	100
				246	100

[7] AMR genes identified by ResFinder analysis. Recovery indicated as percentage of recovered colonies on agar plates that are positive for either shigatoxin gene (*stx1, stx2*) and intimin (e*ae*) through detection via STE PCR. Uninoculated samples from each food commodity were included with 0% recovery achieved.

3.3 Testing new sets of Antimicrobial Resistance Gene predictions and antibiotics for recovery of various STEC strains from ground beef

A total of 9 different antibiotics were tested for their effectiveness in custom selective enrichment of STEC from ground beef using multiple antimicrobial resistance gene determinants from 5 different non-O157:H7 STEC strains; O111:H11, O111:H8, O121:H19, O103:H2, O2:H25 (table 1). The strains were chosen from a limited set of previously sequenced outbreak related strains at the CFIA, and the antibiotics were selected to match the most common AMR traits identified within our model organisms. Concentrations for antibiotics were selected as decimal figures (i.e. 10 or 100 ug/mL) based on nearest breakpoint data (CLSI, 2018). Regular MIC values from standard protocols require stringent sample preparation and homogeneity which is not possible with a heterogenous food sample. Breakpoint values used to determine experimental antibiotic concentration may not reflect the most ideal concentration for recovery of STEC, as these values were chosen for the sake of simplicity and universal application. Strain information is shown in table 1 and recovery data in table 4.

Background microbiota levels for ground beef samples ranged from $2.6x10^3$ CFU/g to $8.0x10^7$ CFU/g. The inoculated strain was not detected after enrichment in the absence of antibiotics (table 4). Recovery of target STEC with antibiotic supplementation was successful in most cases regardless of genomic predictors for resistance. For certain antimicrobial classes, where a single gene predicts resistance to multiple antibiotics within that class, significantly different recoveries can be produced, and each antibiotic should be validated individually. Kanamycin resistance in strain OLC455 predicted from *aph(3')-Ia* yielded an average of 32% recovery, whereas Neomycin resistance predicted

by the same gene within the same strain yielded a much higher recovery at 94%. Therefore when *aph(3')-Ia* is predicted in an organism, Neomycin would be used preferentially for its recovery. All other genomic predictions and corresponding antibiotics used in table 5 were able to generate recovery of STEC at or near 100%, and would be recommended for use in custom selective enrichment of STEC when these specific AMR genes are identified.

Table 5: Recovery of non-O157:H7 STEC from fresh ground beef. Background microbiota levels for ground beef samples ranged from 2.6x103 CFU/g to 8.0x107 CFU/g. Recovery values are shown from two biological replicates per antimicrobial prediction. Strain information can be found in table 2.

Antibiotic	AMR marker	STEC strain	Inoculum (CFU)	Recovery (%)
Trimethoprim (100ug/ml)	*dfrA17*	714	15	83, 100
	dfrA12	792	14	92, 96
Gentamicin (100ug/mL)	*aac(3)*	714	15	21, 58
Kanamycin (100ug/mL)	*aph(3')-Ia*	455	20	25, 38
Neomycin (100ug/mL)	*aph(3')-Ia*	455 714	20 28	92, 96 100, 100
Streptomycin (100ug/mL)	*aph(6)-Id*	455 969 1337	20 30 42	67, 75 96, 100 100, 100
	aph(3'')-Ib	969 1337	30 42	96, 100 100, 100
Sulfadiazine (100ug/mL)	*sul1*	714	17	100, 100
	sul2	714 969	17 29	100, 100 88, 96
Chloramphenicol (10ug/mL)	*catA1*	714	15	38, 88
	floR	1337	42	88, 100
Florfenicol (10ug/mL)	*floR*	792 1337	14 42	100, 100 98, 100
Penicillin G (100ug/mL)	*blaTEM*	714 969	17 29	64, 96 79, 83
	blaCMY	792	14	8, 12

These results demonstrate that custom selective enrichment is an effective tool for recovery of STEC in food outbreak scenarios, where AMR features are present in the target pathogen. Selective enrichment using antibiotics achieved positive recovery of STEC for all tested antibiotics in every tested food commodity. Even when low levels of

recovery were produced, there was a marked improvement over not using any antibiotic at all. In each scenario without antibiotics, the inoculated target strain was not detected. This highlights the extremely competitive growth conditions in microbiologically complex food matrices and supports development of a new method of recovery to address the lack of a universal enrichment medium for STEC. Variable recoveries of STEC were obtained when antibiotics were added to the enrichment, but all were able to generate net improved recoveries of STEC from a contaminated food sample, which is more than sufficient in a food safety investigation where only 1 viable cell is required for action. The nature of the AMR-gene specifying resistance to a particular antibiotic was shown to be of importance. Recovery of STEC was limited to 10% when Penicillin G resistance was predicted by presence of the beta-lactamase *blaCMY*, however, a separate strain with a different beta-lactamase (*blaTEM*) was recovered at 96% using the same antibiotic. Thus, Penicillin G would not be recommended as a supplement in custom enrichment when *blaCMY* is indicated but would be for *blaTEM*.

The mechanism of action of the antibiotic should also be taken into consideration when interpreting recovery values. Bactericidal antibiotics like penicillin and streptomycin kill bacteria, whereas bacteriostatic antibiotics like sulfadiazine prevent bacterial replication, but do not kill individual cells (Nemeth et al., 2015). Recovery of non-target colonies could be due to the bacteriostatic nature of the antibiotic being used, where aliquots of the supplemented enrichment broth are transferred to un-supplemented MacConkey agar plates for recovery of individual colonies. We chose to leave the agar plates un-supplemented to promote colony growth after enrichment, but also to create the least variation from current methodologies, which do not supplement their differential

agar media with antibiotics (Blais et al., 2014). Once removed from the antibiotic, it is possible for non-target colonies to resume replication. Therefore, the presence of non-target colonies does not guarantee that those colonies are resistant to bacteriostatic antimicrobials used in the enrichment. Inclusion of antibiotics in the agar media as well as the enrichment both would continue to suppress non-target bacterial growth after enrichment but was not needed to generate increased recovery. When non-target colonies are present in bactericidal antibiotic treatments, acquired resistance to that antibiotic is likely present.

3.4 Impact of background microbiota composition

Recovery of STEC below 100% in previous sections indicates the persistence of native microbiota through the selective enrichment process. These non-target organisms are evidence of AMR traits circulating within the native microbiota of the food sample, which allow them to survive in the custom enrichment environment and compete with the target organism when the appropriate antibiotic is indicated. We aimed to determine what species of bacteria are directly competing with the pathogenic STEC in our enrichment cultures by identifying the non-target organisms using a VITEK system. The VITEK system is used for automated microbial identification and antibiotic susceptibility testing, after isolation of a primary organism (Pincus, 2010). Individual non-target colonies (i.e. colonies negative in STE PCR screening) previously isolated from aliquots of enrichment broths in section 3.2 were selected as they represent direct competition to our target organisms.

The microbial community identified by VITEK was variable based on both food commodity and antibiotic treatment (Table 6). Spinach samples showed an increased diversity in isolates obtained from different antibiotic treatments, whereas ground beef saw a more uniform distribution. TMP and CHL enrichment yielded mostly *Pseudomonas* species in spinach, whereas treatment with GEN saw competition reduced to a single species; *Delftia acidovorans*. *Serratia liquefaciens* group was the most predominant species recovered from ground beef, regardless of antibiotic treatment. While the small sample size is acknowledged, these results correlate with our AMR data generated from the NCBI database (figure 5), where the prevalence of AMR shown by NCBI indicates these organisms may grow under our conditions. VITEK microbial analysis of ground beef and spinach enrichments indicated that *Pseudomonas* species were most prevalent in our samples, and most of the *Pseudomonas* isolates on the NCBI database have genomic determinants for aminoglycoside and beta-lactam resistance, which would explain recovery of STEC below 100% when enriched with antibiotics in those classes.

Table 6: Microbial Identification of native colonies in spinach and ground beef samples after selective enrichment. Bacterial colonies identified by VITEK (Pincus, 2010).

Food Commodity	Antimicrobial Treatment	Recovered Species
Spinach	TMP (100ug/mL)	*Pseudomonas putida* *Kluyvera intermedia* *Pseudomonas fluorescens* *Lelliotta amnigena* *Pseudomonas aeruginosa*
Spinach	GEN (100ug/mL)	*Delftia acidovorans*
Spinach	CHL (10ug/mL)	*Pseudomonas aeruginosa* *Pseudomonas putida*
Ground Beef	TMP (100ug/mL)	*Serratia liquefaciens* *Pseudomonas fluorescens* *Hafnia alvei*
Ground Beef	GEN (100ug/mL)	*Serratia liquefaciens* *Pseudomonas fluorescens*
Ground Beef	CHL (10ug/mL)	*Pseudomonas putida* *Serratia liquefaciens* *Ewingella americana* *Pseudomonas fluorescens*

3.5 Effect of food storage related stress

Storage conditions for raw foods are designed to prevent pathogen contamination, with products often being stored at 4°C. These are not ideal temperature conditions for bacteria, which limits their growth and can induce stress into the organism. The scenario in which recovery of a low-level STEC contamination event is hindered due to the less-than-ideal conditions provided by these storage environments was considered. Real-world

storage conditions were simulated for an artificially contaminated food sample to determine if physiological changes produced by a higher stress environment will hamper recovery of the pathogen. Inoculated ground beef samples were equilibrated at 4°C for 72 hours prior to enrichment to simulate real-world conditions, non-equilibrated samples were enriched immediately after inoculation.

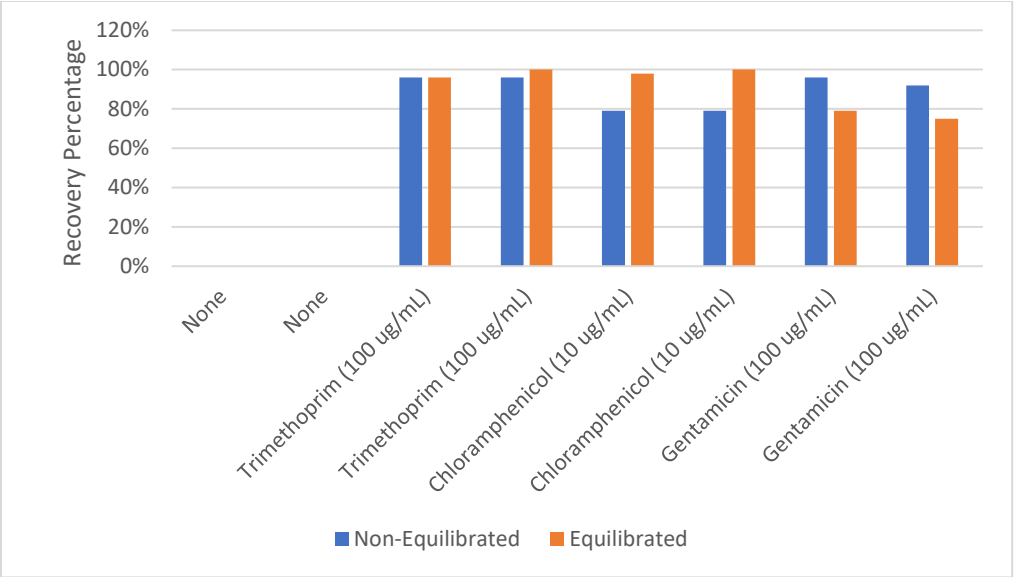

Figure 6: Recovery of STEC strain OLC-714 in ground beef. 25g portions of ground beef inoculated with 24CFU STEC: (blue) enriched immediately; (orange) enriched after storage for 72h at 4°C.[8]

Without antibiotic supplementation, STEC were not detected in either treatment. Enrichment with GEN yielded 94% recovery of STEC in un-equilibrated conditions, recovery decreased to 77% in equilibrated conditions. Conversely, enrichment with CHL had a higher recovery from equilibrated conditions (98%) than un-equilibrated (79%). Enrichment with TMP yielded over 96% recovery of STEC in both conditions. Our

[8] Enrichment consisted of supplementing mTSB with one of Trimethoprim (100ug/mL), Gentamicin (100ug/mL), or Chloramphenicol (10ug/mL).

results indicate that storage conditions at 4°C for produce and raw meats does not limit the ability of selective enrichment to recover a target pathogen (figure 5).

3.6 Alternative selective enrichment options

Antibiotic resistance in STEC is not universal, and there could be instances where no identifiable antibiotic resistant traits are found in an outbreak strain as determined through AMR analysis of OLC and NCBI datasets. We aimed to identify other genomic predictors for antimicrobial resistance to bolster the catalogue of useable antimicrobials for custom enrichment. For the recovery O157:H7 STEC, decreased susceptibility to Tellurite and Novobiocin antimicrobial agents have been leveraged for its recovery from a food sample, but resistance to these antimicrobials is variable outside of the O157 serotype (Gill et al., 2012; Orth et al., 2007; Valková et al., 2007). The FDA's Bacteriological Analytical Manual (BAM) indicates 10ug/mL Novobiocin and 0.8ug/mL Potassium Tellurite (K2TeO3) to be supplemented for assisted recovery of O157:H7 STEC (United States, 1984). To determine if resistance to these antimicrobials can be accurately predicted for non-O157 STEC, a panel of 10 non-O157 STEC and 2 O157 STEC were selected to test for their Potassium Tellurite and Novobiocin MIC's (Table 7). The panel was chosen based on the presence of genomic markers indicating potential tellurite resistance, previously described as the *ter* gene cluster in O157:H7 *E. coli* (Orth et al., 2007). Novobiocin resistance is mediated through the presence of SNP's in related genes, which were determined for each strain after the panel was chosen.

Table 7: Panel of 12 STEC strains selected for Minimum inhibitory concentration (MIC) testing of Tellurite. [9]

Strain	**Serotype**	**Tellurite Resistance Genes**
803	O157:H7	*terZ, terW*
1358	O157:H7	*terZ, terW*
2243	-:H26	*terZ, terW*
2364	O176:H4	-
2383	O153/O178:H19	*terD, terZ*
2524	O118/O151:H2	*terD, terZ, terW*
2526	O55:H12	*terZ, terW*
3374	O9:H30	*terZ*
3407	O176:H4	*terD, terZ*
3456	O176:H4	*terD, terZ*
3483	-:H19	*terD, terZ*
3504	O8:H25	*terZ, terW*

3.6.1 Tellurite

A broth microdilution experiment performed on 12 STEC strains, of which 2 were O157:H7, gave inconclusive results for potassium tellurite. 11 of the 12 STEC strains contained at least one constituent of the *ter* gene cluster, which was expected to confer

[9] Panel was selected based on presence of Tellurite resistance Genes identified by GeneSeekr; https://olc-bioinformatics.github.io/redmine-docs/analysis/geneseekr/ . Strains obtained from CFIA's Ottawa Laboratory (Carling) Bacterial Culture Collection.

resistance to tellurite based on previous studies (Kormutakova et al., 2000; Whelan et al., 1995). While the mechanism is not completely understood, the components of the *ter* cluster are thought to form a metal-binding multi-protein complex that associates with the cell membrane. This complex can result in membrane-proximal reduction of tellurite causing its crystallization at the cell membrane, limitng its toxicity (Anantharaman et al., 2012). While all 11 *ter* positive strains had MIC values equal or above 6 µg/mL, the *ter* negative isolate also had an MIC of 6µg/mL. Additionally, MIC values were not consistent for *ter* positive strains; ranging from 6-50µg/mL. The *ter* operon is not the only means for bacterial tellurite resistance, and we suspect there are other factors contributing to the tellurite resistance patterns seen in our STEC panel. The mechanism of tellurite toxicity is not completely understood, however, two models have been proposed. Firstly, it was argued that the ability to act as a strong oxidizing agent over a variety of cell components contributed to its toxicity (Summers & Jacoby, 1977; Turner et al., 1999). More recently, evidence has been provided that tellurite exerts its toxicity through intracellular generation of reactive oxygen species (Borsetti et al., 2005; Rojas & Vásquez, 2005; Tantaleán et al., 2003). Other metabolic pathways which help to reduce ROS could have been present in our strains and contributed to a lack of sensitivity. Production of oxidase and catalases have been reported in *E. coli,* where they contribute to removal of ROS species (Khademian & Imlay, 2017; Wan et al., 2017). Resistance to potassium tellurite can also be influenced by presence of efflux pumps (Turner et al., 1992a). While the presence of these pumps can be confirmed genomically, such as the arsenical ATPase which can mediate resistance to tellurite (Turner et al., 1992b) the level to which they are expressed in a cell is much more difficult to discern. Small changes to

upstream elements of the related genes could result in increased or decreased expression of tellurite-efflux related pumps. While these changes could be identified genomically, epigenetic changes that influence expression would not be identified when doing sequence alignments and could result in increased phenotypic resistance to tellurite.

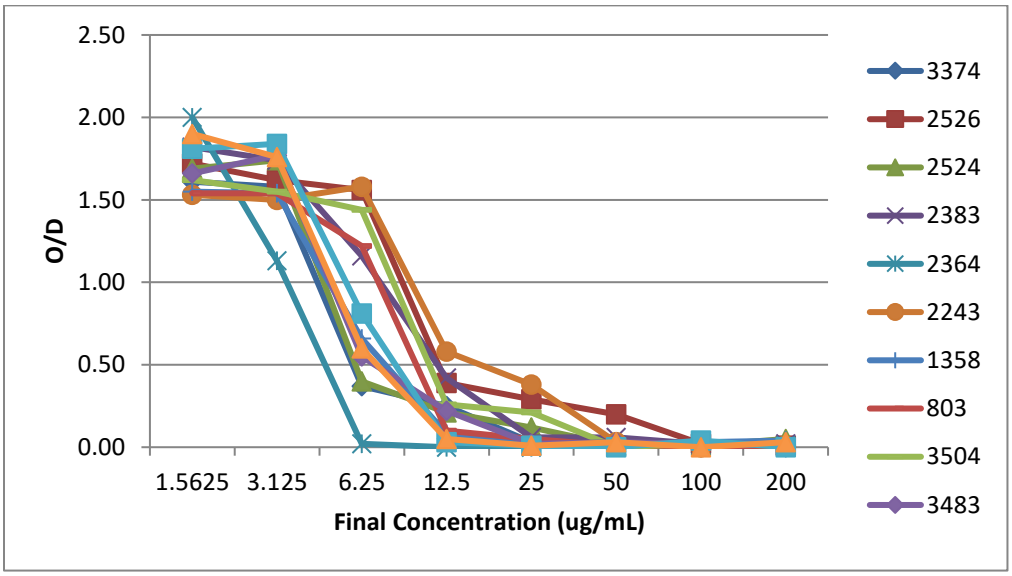

Figure 7: Minimum Inhibitory Concentration for STEC strains in doubling concentrations of Potassium Tellurite (ug/mL). Strain information can be found in table 5.

3.6.2 Novobiocin

Novobiocin resistance can be mediated through the presence of non-synonymous SNP's in DNA gyrase (*gyrB*) and topoisomerase IV (*parE*) related genes (Breines et al., 1997; Yoshida et al., 1991). Given the range of novobiocin MIC values generated from our panel (12.5µg/mL to 100µg/mL) (figure 8) the lack of non-synonymous SNP's in *gyrB* and *parE* genes was unexpected (table 8). The highest Novobiocin MIC belonged to strains OLC2526 and OLC2524 at 200 ug/mL. The lower end of Novobiocin MIC's was shared by three strains at 25 µg/mL.

Table 8: Non-synonymous Single Nucleotide Polymorphisms in Novobiocin related genes of STEC[10]

	parE			***gyrB***		
Strain	Codon Position	Reference Codon	Sample Codon	Codon Position	Reference Codon	Sample Codon
803	-	-	-	-	-	-
1358	405	ACC (thr)	TCC (ser)	-	-	-
2243	-	-	-	-	-	-
2364	-	-	-	-	-	-
2383	-	-	-	-	-	-
2524	-	-	-	-	-	-
2526	-	-	-	-	-	-
3374	-	-	-	-	-	-
3407	-	-	-	-	-	-
3456	-	-	-	-	-	-
3483	-	-	-	-	-	-
3504	-	-	-	-	-	-

Non-synonymous SNP's in Novobiocin related genes for the tested strains were tracked. *gyrB* genes were identical at the amino acid level across all strains. *parE* had one non-synonymous mutation in strain OLC1358 (O157:H7) resulting in a Threonine to Serine substitution (ACC – TCC) at position 405 (table 8). The remaining strains had no variation between them at the identified genes. The prospect of multiple gene copies could explain the inability to detect SNP's that would be responsible for the variation in novobiocin resistance of our STEC panel. The analysis of our strains would have only

[10] Reference nucleotide sequences for alignments located on NCBI; *E. coli str. K-12 MG1655*. Gene ID: *parE* – 947501, *gyrB* – 948211.

identified one of the genes, regardless of any disparity between the two. In retrospect, analysis of the number of reads could give insight into this issue, as the number of reads would be significantly larger for multiple copies of a single gene.

It is crucial to have an accurate genomic predictor for resistance to avoid false negative detection of STEC, as un-informed use of antimicrobials for recovery has potential for controversy (Vimont et al., 2007). Our results suggest the currently used concentrations of 10 ug/mL Novobiocin in STEC recovery protocols could potentially be inhibitory to some STEC strains. This potentially dangerous situation is avoided with the prospect of selective enrichment, which through predictive genomics would exclude the use of Novobiocin in sensitive strains.

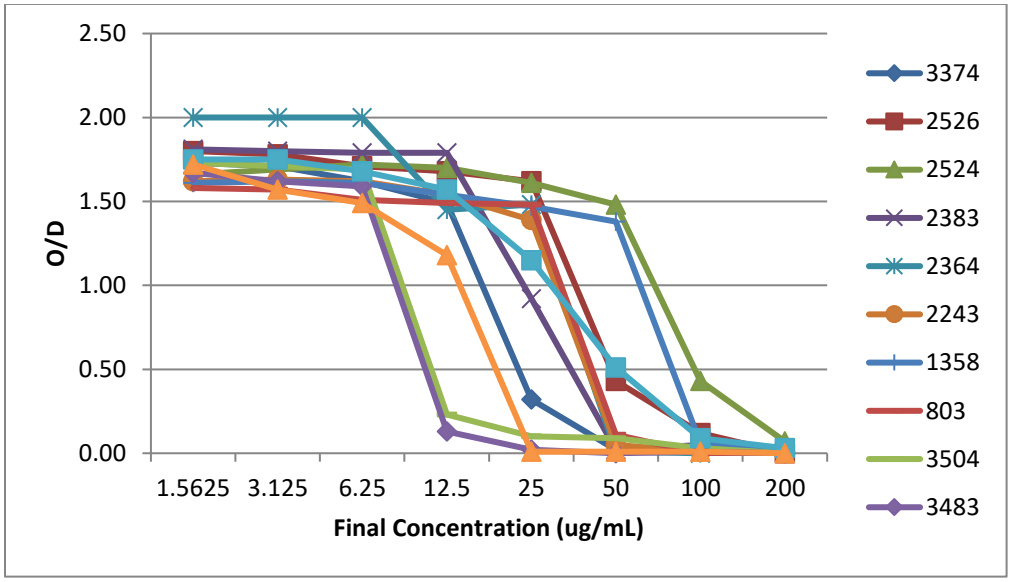

Figure 8: Minimum Inhibitory Concentration for STEC strains in doubling concentrations of Novobiocin (ug/mL). Strain information can be found in table 5.

Custom selective enrichment is intended to be applied during food safety investigations to aid in the timely recovery and identification of pathogenic bacteria from foods. In these situations, the method for source identification and traceback needs to be both accurate and time-sensitive, as every day that passes incurs increased risk of illness to the general public. The custom selective enrichment method utilizes a readily ascertainable feature associated with particular strains of interest to reliably recover them from contaminated foods. The method also uses the same enrichment broth and plating media that is currently implemented in Canada's laboratory procedures for the microbiological analysis of foods (MFLP-52). In fact, the only additional reagents that would be required would be the antimicrobials which have been previously catalogued for use. The genomic information regarding selection of the antimicrobials is analyzed before sample reception and supplementation takes no longer in the described method compared to current methods. In the event of an outbreak investigation and to have the most confidence in our result, the suggested action would be to process two sub-samples in parallel. One would use selective enrichment with the indicated antibiotic, and another run in parallel with conventional methods.

3.7 Limitations & Future Directions

Although this approach shows promise, there is more work to be done. Accurate comparative analysis of AMR traits will help to bolster the method. VITEK is useful for identifying individual colonies and antibiotic susceptibility, but it is reliant on the recovery of individual colonies on agar media and may be prone to selection bias in that regard. Metagenomic analysis of high-risk food commodities would allow for more

accurate identification of all species in the sample environment and limit recovery bias (Kergourlay et al., 2015). This would allow for more accurate prediction of AMR in the native microbiota and strengthen our ability to inform selection of the most useful antimicrobial. More work should be done to validate determinants for tellurite and novobiocin resistance, as they are already being used to isolate certain STEC serotypes. Tellurite is known to promote generation of ROS, investigation into ROS reducing processes and enzymes such as cysteine metabolism and production of catalases may allow for prediction of tellurite resistance and allow for its use in custom selective enrichment.

Lastly, while this method was able to identify several strong candidates for use in recovery of STEC, a more comprehensive catalog of validated resistance determinants is needed to increase the potential of the method. This means a wider variety of antibiotics and additional antimicrobials outside of main-stream antibiotics should also be considered; any compound capable of limiting bacterial growth with an identifiable genomic marker for predicting resistance is a candidate for selective enrichment. One possible avenue is exploitation of rare sugar fermentation in bacteria, where if predicted genomically, would allow for an enrichment environment to be formulated using the rare sugar as the sole carbon source, thus promoting growth of that indicated strain. In conclusion, results of the current study show genomically informed custom selective enrichment is valuable in the reduction of false-negative detection of STEC and in support of overall outbreak management.

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