

Surface directed monoclonal antibodies against STEC aid in the reduction of pathogen detection times from food and water

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Abstract

The diagnostic methods implemented at the Canadian Food Inspection Agency for the detection of Shiga toxin producing *E. coli* (STEC) are time consuming and tedious, taking up to 5 days before a positive sample can be confirmed. The goal of this project was to streamline the detection procedure for serogroup O157 and 6 important non-O157 serogroups of STEC. Following a short enrichment step (4-6 hrs), two approaches were considered: (1) the filtration of enrichment culture through a gradient of filtration membranes (decreasing pore sizes), followed by capture using specific monoclonal antibody (mAb)-coated Dynabeads, and detection via fluorescence microscopy, (2) the addition of enrichment culture into a flow through system consisting of a column packed with large polystyrene beads ($\geq 100 \mu\text{m}$) coated with specific mAbs for capture. The results indicate that the filtration approach can only be applied to simpler food matrices. However, at least 100 CFU of the target STEC could be recovered using the filtration system following 4 hrs of enrichment of these matrices spiked with $\leq 15\text{CFU}$ of the target STEC. Similar capture results were obtained in the second approach using specific mAbs immobilized on covalently coupled protein G polystyrene beads and diluted enrichment media. A combination of these strategies together with immunofluorescence microscopy (IMS) and polymerase chain reaction (PCR) could provide diagnostic laboratories with a means to confirm a positive sample within 2 days of testing.

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List of abbreviations

STEC: Shiga toxin producing *E. coli*

EHEC: Enterohemorrhagic *E. coli*

CFIA: Canadian Food Inspection agency

IFM: Immunofluorescence microscopy

PCR: Polymerase chain reaction

mAb: Monoclonal antibody

FNAB: 1-fluoro-2-nitro-4-azidobenzene

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1.1 *ESCHERICHIA COLI*

Escherichia coli is a Gram negative, rod shaped bacterium that was first described in the late 1800s by Dr. Theodor Escherich (1, 2). It is a ubiquitous species found in various environments, however its members are mostly found as commensals in the gastrointestinal (GI) tracts of warm blooded animals (3, 4). Strains of *E. coli* are classified into different serotypes based on the variations found in their lipopolysaccharides (LPS) (O-antigen), and flagellin proteins (H- antigen). 173 O antigens, and 56 H antigens have been identified to date. In nature different combinations of these antigens can occur, and as a result it is thought that over 50000 serotypes exist (5). Despite these large numbers only a few serotypes have been frequently associated with human disease. These pathogenic *E. coli* can cause intestinal or extra-intestinal infections, and the group causing intestinal infections have been further classified into 6 pathotypes based on their genetic and pathogenic features: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin producing *E. coli* (STEC) (4, 6). The work proposed in this thesis will be focussed on the development of improved methods for the isolation, detection and recovery of major food and water borne STEC. The pathogenesis, epidemiology, and detection of STEC will be discussed below, thereby giving credence to the work proposed herein.

1.2 SHIGA TOXIN PRODUCING *E. COLI* (STEC)

Pathogenic *E. coli* that express the Shiga 1 toxin (*stx1*) and/ or Shiga 2 toxin genes (*stx2*) are classified as Shiga toxin producing *E. coli* also known as STEC. In 1977, Konowalchuk *et al* reported that the production of toxins that were cytotoxic to Vero cells helped to differentiate these pathogenic STEC from others (7). It was later demonstrated by O'Brien *et al* that one of these toxins was structurally similar to the Shiga toxin produced by *Shigella dysenteriae* type 1 (8, 9).

Since these discoveries, the terms VTEC (Verotoxin-producing *E. coli*) and STEC have been used interchangeably to describe strains of *E. coli* that produce these toxins. However to avoid confusion these strains will be referred to as STEC henceforth. STEC cause GI infections with the potential of becoming systemic. Infection usually begins with watery diarrhea in conjunction with abdominal pain and nausea which may or may not develop into bloody diarrhea. Hemolytic uremic syndrome (HUS) is a more severe outcome of STEC infection developing between 2-14 days after the onset of infection, it is characterized by renal damage where 50% of those affected will suffer permanent renal impairment, while 10% will either die from the disease or suffer permanent renal failure with the elderly and very young being at most risk (10). Enterohaemorrhagic *E. coli* (EHEC) are a subset of STEC and are usually associated with HUS and more severe clinical outcomes. Generally the development of HUS has been linked to the presence of a specific set of virulence genes that give rise to adhesion and effacement lesions on enterocytes, however EHEC have been isolated lacking these genes, suggesting that HUS may result from the combined action of multiple virulence genes, the mechanism of which is not clearly understood (6, 11). In this study the focus will be on the top 7 EHEC serogroups (O157, O26, O45, O103, O111, O121, and O145) that have been reported to cause severe illness in humans in North America (12, 13).

1.3 GENOMIC BASIS OF VIRULENCE

E. coli can have genomes that greatly vary in size with differences of up to a million base pairs being reported in pathogenic strains compared to non-pathogenic strains. The genome can be divided into two parts; the first consists of a conserved set of genes known as the core genome, and the second consists of a more dynamic gene pool (14). The plasticity of the genome is afforded by the ability of *E. coli* to lose and gain genetic material through processes such as transformation, conjugation and transductions via mobile genetic elements (15, 16). These mobile elements have

been shown to be essential for the virulence traits observed in pathogenic strains like STEC as most virulence genes can be found on pathogenicity islands or on large plasmids. Examples of note of some chromosomally encoded virulence genes are: (i) the locus of enterocyte effacement also known as LEE which is found in a 35 kb gene cluster located on a pathogenicity island of the genome of some STEC (17), and (ii) *stx* genes which are chromosomally integrated through lysogenic lambdoid bacteriophages (18). Some STEC also carry large plasmids that contain virulence genes such as enterohemolysin, and extracellular serine protease among others which contribute to its pathogenicity (19-22). These chromosomally encoded virulence factors along with the genes encoded on acquired plasmids make STEC a formidable human pathogen.

1.4 TRANSMISSION

STEC are usually found in the GI tract of ruminants like cattle, sheep, and deer which serve as major reservoirs for these pathogens (23-25). The most significant route of transmission of these pathogens is via the fecal–oral route, and many instances of illness have stemmed from the consumption of contaminated food or water, direct contact with carrier animals, and symptomatic and asymptomatic patients (26-29). It has been reported that the infectious dose and clinical outcomes vary due to the virulence genes harbored by the pathogen (30), and immune status of the individual (31, 32). Unlike other pathogenic *E. coli*, STEC are unique as they have very low infectious doses, with doses as low as 1 CFU/ 10g of meat being reported (33). In addition viable but non culturable STEC can be found on food matrices and can continue to produce Shiga toxin, however the reports discussing infectious doses do not take these cells into account and their contribution to illness is therefore not clear (34). Cattle that serve as a reservoir for STEC have been reported to shed large numbers of STEC in their feces (up to 10^6 CFU/ g of feces) (35) that then have the ability to persist in the environment for prolonged periods of time (months to up to

a year) (36, 37). Food and water can become contaminated with STEC upon contact with tainted feces. It has been suggested that meat can become contaminated with STEC during the slaughtering, dehiding, or processing of the carcass (38, 39). Tainted manure, irrigation water, and farm runoff have been thought to contribute to the contamination of food like vegetables and water sources like lakes, rivers and underground water (well water) (40-42). Interestingly, though over 20% of the beef tested in the US test positive for *stx* genes (PCR); only a small proportion of those isolates pose a threat to public health (43).

1.5 MECHANISMS OF VIRULENCE

Though the production of Shiga toxin is a defining characteristic of STEC, the presence of these genes alone is not sufficient to cause disease. However the acquisition of these genes together with the presence of a background genome containing complementing virulence factors confers the broad pathogenic profile caused by STEC infection (44, 45). Distinct mechanisms come into play to establish infection within the host's GI tract; some of the more pivotal mechanisms involved will be described below.

1.5.1 RESISTANCE TO ACIDIC ENVIRONMENTS

One of the first host defenses that GI pathogens have to overcome is the acidic environment encountered in the stomach, followed by exposure to weak acids produced by commensal flora in the small intestine which can be lethal to invading organisms (46, 47). Despite being exposed to such harsh conditions many GI pathogens are able to persist and cause infection. Three mechanisms have been reported to be essential for acid resistance in these pathogens. These mechanisms are (i) the oxidative acid resistance system that is dependent on the alternate regulatory factor *rpoS* and is expressed in response to stress, (ii) arginine dependant and (iii)

glutamate dependant acid resistance systems. It has been suggested that a combination of these different mechanisms can lead to the high level of acid resistance seen in successful GI pathogens (48). Studies that compared the acid resistance of non-pathogenic *E. coli* and STEC that cause human disease demonstrated that there were no major differences in the resistance conferred by the glutamate and oxidative resistance systems to both groups. However the arginine dependant system seemed to provide STEC with an edge over their non-pathogenic counterparts at pH 2.0 (46). Previous reports that studied other GI pathogens have shown that there is a clear correlation between acid resistance and low infectious doses, which suggests that the low infectious doses reported for STEC could be directly related to their ability to resist the acidic environment of the host's GI tract (49- 51). Furthermore it has been hypothesized that STEC can be exposed to acidic environments within the ruminant gut where acid resistance mechanisms are activated. Once activated, the resulting acid resistance can persist for long periods of time under cold storage conditions. As a result it is likely that STEC derived from the ruminant gut that then go on to contaminate food and water are already primed to overcome the acidic environment of the host's GI tract (46).

1.5.2 ADHESION

GI pathogens that are unable to adhere to intestinal mucosa following their passage from the stomach are removed from the system via peristalsis (6). Pathogens like STEC however utilize adhesion structures which allow attachment to the mucosa thus enabling colonization. Adhesion structures have been well characterized in other pathogenic *E. coli* (52), however the role that adhesins like fimbriae and outer membrane proteins (OMP) play is still unclear in the case of clinically significant STEC (53-55). A recent study aimed at correlating adhesion and the presence of the virulence genes with a potential role in adhesion suggested that the presence of the *eae* gene

strongly correlated with the ability of STEC to adhere to epithelial cells (56). The LEE pathogenicity island of EHEC and EPEC carry genes that code for *eae* which is an outer membrane protein (intimin), effector proteins, and a type three secretion system (TTSS) (57). One of the effector proteins is the translocated intimin receptor (Tir), which is translocated into the host cell via the TTSS followed by its insertion into the host membrane where it serves as a receptor for intimin. This allows for intimate attachment of the pathogen to the host epithelial cells (58).

1.5.3 ATTACHMENT AND EFFACEMENT

Following attachment to the host cell via the Tir-intimin interaction, a number of other effectors come into play leading to some of the clinical manifestations observed during a STEC infection. One of the key characteristics of EHEC is their ability to form A/E lesions on GI mucosal epithelia. A/E lesions are caused by pedestal formation beneath the site of Tir-intimin interactions and involve a multistep process (59). Depending on the serotype of STEC being studied this pedestal formation may be initiated in a Tir phosphorylation dependant, or independent manner. In the phosphorylation independent approach, the effector protein EspF is recruited by Tir and mediates actin polymerization leading to the formation of the pedestal (60). In other instances, the phosphorylation of Tir leads to a cascade of interactions which ultimately leads to the remodelling of actin filaments forming the pedestal (61). The attachment and effacement step is followed by the action of different effector proteins that negatively affect the integrity of tight junctions (62), water absorption, and ionic exchange, which leads to the distinctive localized characteristics of a STEC infection which include lesions, diarrhea, and the disruption of epithelial microvilli cells (63, 64).

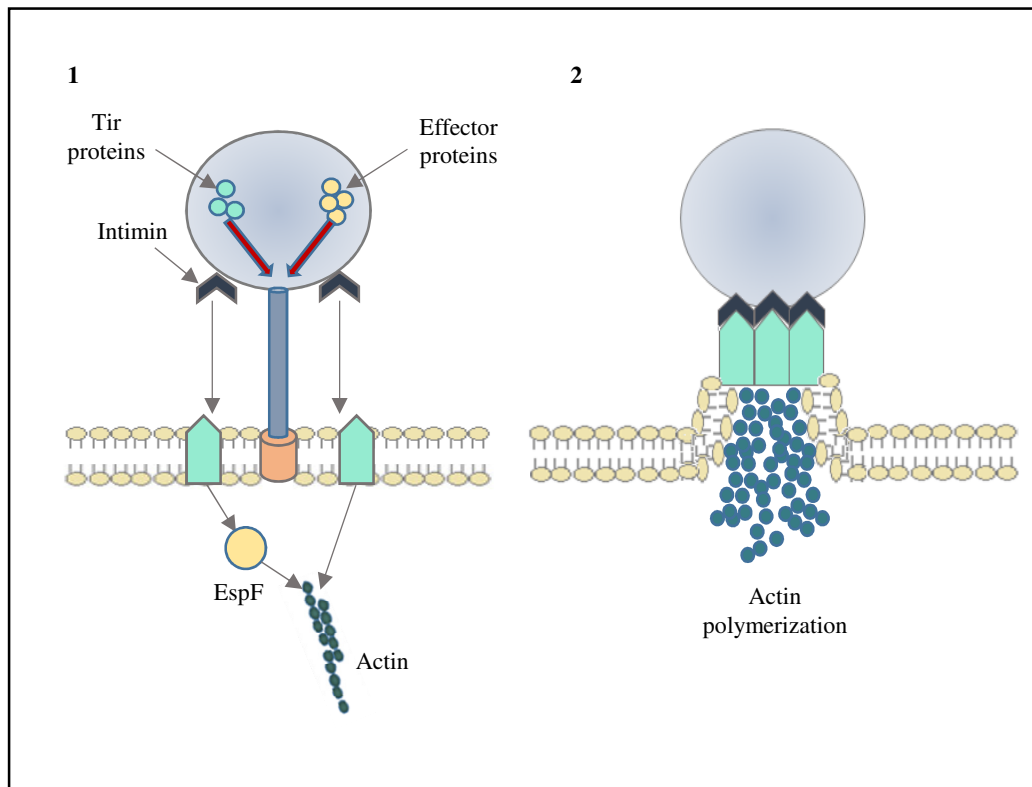


Fig 1: Pedestal formation by EHEC. (1) Upon contact with the host cell, and assembly of the type 3 secretion system, EHEC effector proteins are translocated into the host cell via the TTSS. Tir is translocated into the cell and is inserted into the plasma membrane of the host where it serves as a receptor for intimin (bacterial outer membrane protein). This interaction is followed by a cascade that leads to actin polymerization and can be Tir phosphorylation dependent or phosphorylation independent acting through the effector protein EspF. (2) B- Actin polymerization leads to the formation of a pedestal beneath the point of interaction which is characteristic of EHEC infections

1.5.4 SHIGA TOXIN

The *stx* genes are found on lambdoid prophages, and are strictly repressed in the prophage stage (65). However it has been shown that these prophages are induced under conditions that initiate the bacterial SOS response. This induction leads to viral replication and lysis of the bacterial cells which effectively releases Shiga toxin (66), which then can be carried to other locations in the body via the blood stream (67). STEC can carry one or both of the *stx* genes (*stx1*, and *stx2*), and though they share some sequence homology (~56%) at the amino acid level (68), they are immunologically distinct with *stx 2* being the more potent toxin contributing to more severe outcomes in human disease (69, 70). Based on their immunological and amino acid homology, various antigenic variants of these toxins have been described (Stx1a, Stx1c, Stx1d, and Stx2a-g) (71). The homology between the different Stx2 variants ranges from 80-99% with some variants only differing by an amino acid, however this variation has been suggested to be responsible to differences in pathogenesis, tropism, and enzymatic activity (72). The structure of Shiga toxins consist of two main subunits – the enzymatic A-subunit and the receptor binding B-subunit pentamer. The B-subunit pentamer binds to globotriaosylceramide (Gb3) receptors on eukaryotic cells, following which it is endocytosed into the cells either via a clathrin dependent or a clathrin independent manner (73). Once in the cell the A subunit is cleavage by furin creating two fragments: an A1 activated fragment which is tethered to the A2 fragment via a disulfide bond (74). The A1-A2-B complex remains intact as it undergoes retrograde transport from the golgi to the endoplasmic reticulum (ER) where the disulfide bond is reduced freeing the A1 fragment (75). The enzymatically active A1 unit moves into the cytosol where it removes a single adenine residue from the 28S rRNA within the large ribosomal subunit which in turn abrogates the interaction between the ribosome and the elongations factor effectively inhibiting protein synthesis (76). The

removal of the residue from the 28S rRNA leads to the activation of the ribotoxic stress response which elicits the production of chemokines, cytokines, and can lead to the activation of signaling cascades resulting in apoptosis (77). Furthermore the accumulation of improperly translated and folded proteins causes an ER stress response which contributes to apoptosis observed in affected cells and organ damage (78). The Gb3 receptor is differentially expressed in different tissues, as a result Shiga toxins tends to effect those organs/ systems where these receptors are expressed. Notably the Gb3 receptor is expressed by multiple cell types in the kidneys and in the central nervous system in humans, making these systems targets for the toxin, and thus explains the more severe manifestations of STEC infections such as bloody diarrhea and HUS (renal and CNS involvement) (79, 80).

1.5.5 LIPOPOLYSACCHARIDE

On average the surface area of an *E. coli* cell is $6.7 \text{ } \mu\text{m}^2$, with approximately 75% of the surface occupied by LPS. LPS is a bacterial glycolipid that consists of three main domains: the lipid A domain, the core domain, and the oligosaccharide domain. The lipid A domain constitutes the lipid component that is responsible for anchoring LPS to the bacterial cell. It is conserved among Gram negative bacteria, and is the toxigenic component of LPS. The core domain can be further divided into the outer and inner core. The inner core is relatively conserved among bacterial species. A key feature of the inner core is that it is composed of 2-keto-3-deoxyoctonoic acid also known as KDO, which is a conserved motif of LPS. This allows KDO to serve as a target in endotoxin testing of LPS. In addition to KDO, the inner core also contains ethanolamine, and l-glycero-d-manno-heptose (81). The outer core is a more variable domain, with variability being observed between species, while remaining relatively conserved within a species and is composed of repeating hexose sugar motifs (82). The oligosaccharide domain also known as the O antigen domain is

highly variable and immunogenic. It usually forms the outer most portion of LPS and extends into the extracellular environment. It consists of long chains of repeating oligosaccharide subunits composed of up to 5 sugars. 20 different sugars have been identified as forming this domain, which accounts for the immense variability observed within a species. As a result of this variability, classification of *E. coli* into different serogroups target the O antigen domain of LPS (83). It has been reported that LPS plays several roles in the pathogenesis of GI bacterial pathogens. These include the inhibition of phagocytosis, the inhibition of complement driven bacterial cell lysis, the activation of signalling cascades that promote cytokine production leading to inflammation, and the lysis of the host cell through complement activation and the membrane attack complex among others (84, 85, 86). LPS has also been suggested to be involved in bacterial adherence, a few groups sought to determine if the O157 serogroup LPS plays a role in cell adherence in STEC infection. The findings were contradictory since one study reported that antibodies directed against the LPS of O157 were able to prevent adherence in vitro, however another study indicated that LPS deficient mutants exhibited higher adherence profiles (87, 88, 89). A few studies have looked at the synergistic effect of Shiga toxin and LPS in the CNS and the renal systems. LPS has been shown to elicit a chemokine response upon activation of renal tube cells, which leads to fibrin deposition, and this response was found to be enhanced by Shiga toxin. LPS as a result contributes to the renal pathology (renal coagulation and thrombosis) observed during STEC infections, while Shiga toxin contributes to the lethality observed in this organ system. Similarly, work focused on the effects of LPS on the CNS suggest that LPS serves to enhance the progression of CNS associated pathology. Taken together these results suggest that the role of LPS in STEC infections is to elicit a pro-inflammatory response and macrophage infiltration (90, 91).

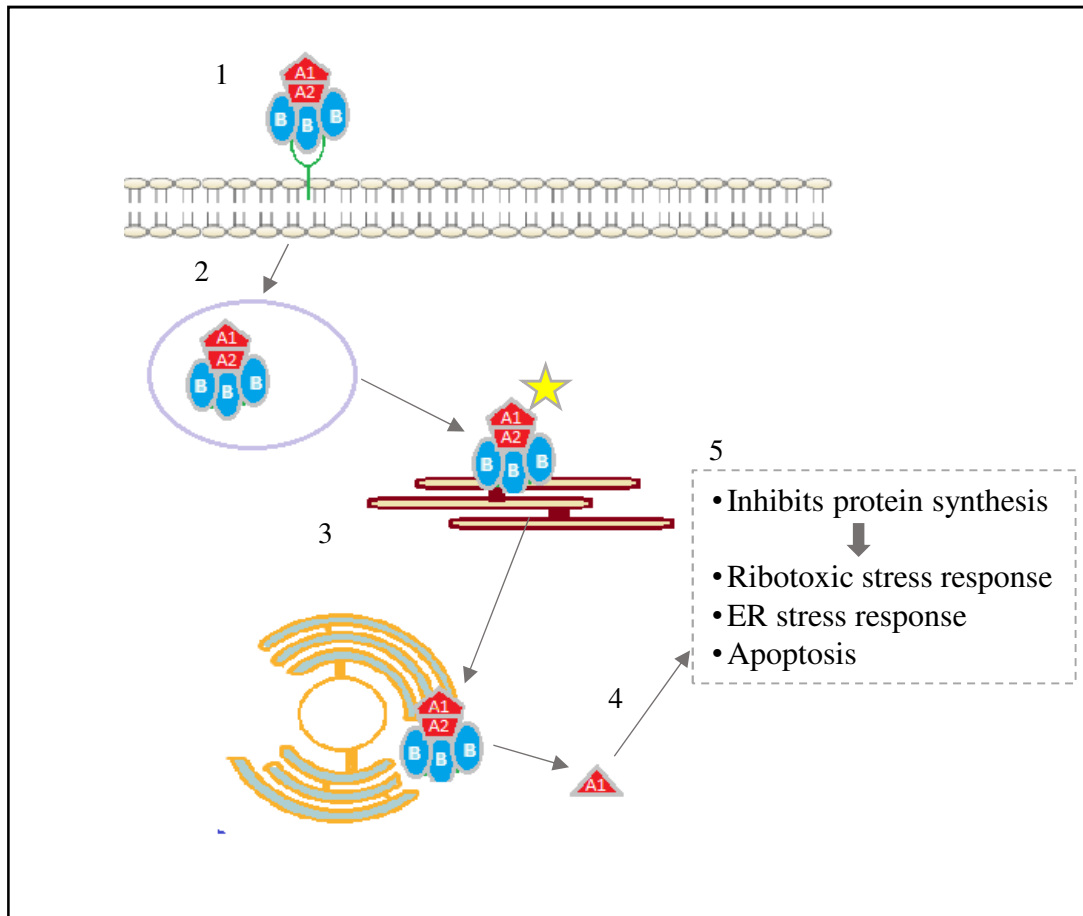


Fig 2: Mode of action of Shiga toxin. Stx consists of two main subunits: the receptor binding subunit–B, and the enzymatic subunit–A. (1) The pentamer subunit B binds to globotriaosylceramide (Gb3), and is endocytosed (2), upon entry into the cell the A subunit is cleaved by furin into two fragments A1 and A2 which remain attached via a disulfide bond. (3) The toxin then undergoes retrograde transport from the golgi apparatus to the endoplasmic reticulum. (4) At the endoplasmic reticulum the disulfide bond is reduced freeing the enzymatic A1 fragment. (5) A1 then acts on its target 28S RNA thereby inhibiting protein synthesis. This in turn leads to a cascade of events that leads to apoptosis of the cell

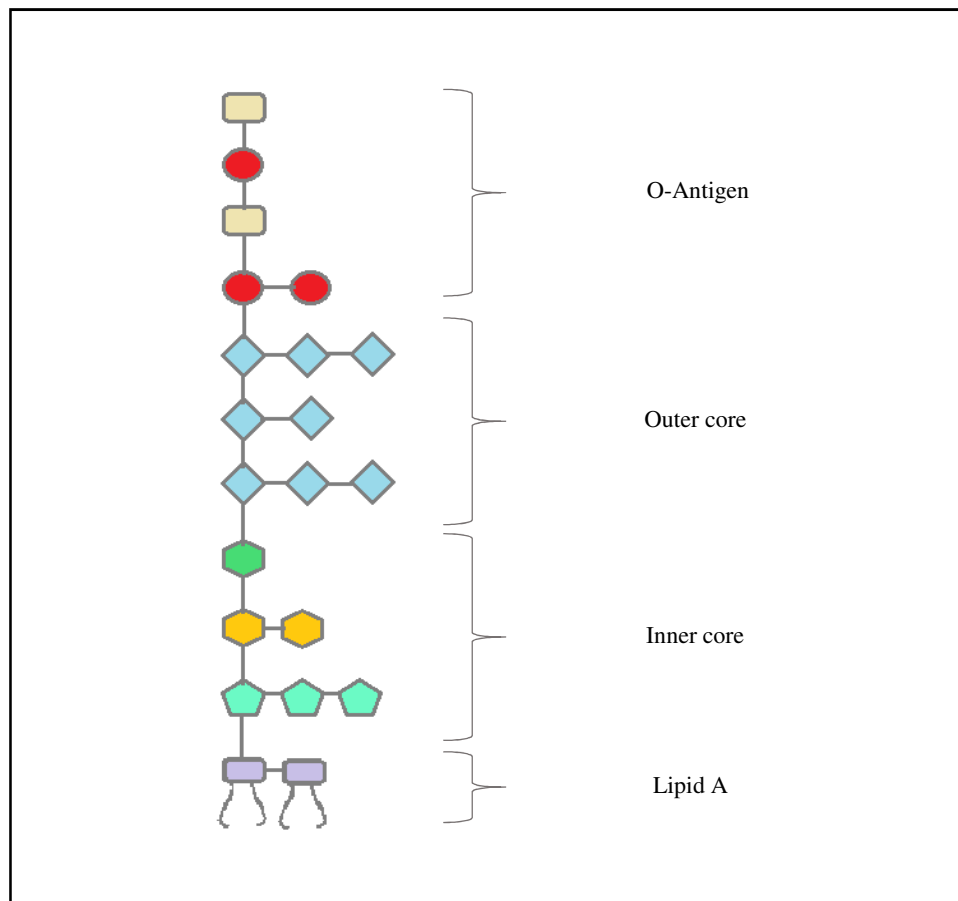


Fig 3: Structure of Lipopolysaccharide. LPS consists of three main domains. The first is the lipid A domain which serves to anchor the LPS molecule to the bacterial cell. The core domain can be further divided into the inner and outer core. The inner core is characterized by the presence of KDO and heptose residues, while the outer core is mainly comprised of hexose residues. The O antigen subunit is the most variable domain and consists of long chains of oligosaccharide subunits.

1.6 INCIDENCE OF STEC RELATED DISEASE

As previously mentioned only a small subset of STEC are associated with human disease, and of this small subset serotype O157:H7 is the most well-known. It was first associated with human disease in the early 1980s and since then has caused multiple outbreaks world-wide (92, 93). In recent years it has become evident that the contribution of non-O157 serotypes to human disease has been underestimated in the past (13). In a study published in 2005, the Center for Disease Control and Prevention (CDC) analyzed over 940 non-O157 clinical isolates from sporadic infections that were collected between 1983 and 2002 and found that a majority of the cases were caused by six serogroups labelled the ““Big six””. These serogroups were identified as: O26, O111, O103, O121, O45, and O145 and caused 22%, 16%, 12%, 8%, 7%, and 5% of the infections reported respectively (94). On the other hand studies have shown that the incidence of O157 related illnesses have dropped dramatically over the years with the US reporting a 42% reduction in incidence in 2011 compared to rates obtained between 1996- 1998 (95). Canadian data also suggests that the prevalence of O157 isolates is on the decline with incidence rates of O157 reducing from 3 cases per 100,000 in 2006 to 1.39 cases per 100,000 in 2012 (96). In 2011, the FoodNet surveillance program reported that there was a higher incidence of non-O157 cases (521) in comparison to O157 cases (463) reported that year in the US (97). Unfortunately not a lot of information pertaining to Canadian non-O157 incidence rates are available since these numbers are not consistently reported to the National Enteric Surveillance Program (NESP) and specific serotypes are usually not identified. However the NESP report published in 2012 suggests that of the 16,934 pathogens reported, 144 were non- O157 *E. coli* serotypes (not typed) (96). The results of these studies along with the information provided by surveillance programs created a case for Food Safety and Inspection Services (FSIS) in the US to push for the development and

implementation of methods to test for non-O157 STEC specifically in food and water. In 2012 the FSIS made it mandatory to test for the “Big six” in meat, and described them as food adulterants. The Canadian Food Inspection Agency (CFIA) is responsible for ensuring that the food and water supply within Canada is safe and free of adulterants. Another important role that the CFIA plays is to maintain healthy trading ties with our trading partners like the US by meeting or exceeding testing requirements. As a result, initiatives are currently being under taken at the CFIA to develop effective and efficient diagnostic tests for STEC including non-O157 serogroups. Different methods that are generally employed for the detection of STEC will be described in the next section, and will include methods currently employed at the CFIA.

Table 1: Examples of STEC outbreak events in Canada from 2009-2014 (adapted from Gideon Infectious diseases of Canada, 2015 edition)

<i>Year</i>	<i>Location</i>	<i>Source</i>	<i>Organism</i>	<i>No. of cases</i>	<i>HUS/Fatalities</i>
2009	Ontario	Onions	O157	235	NA
2009	Ontario	Ground beef	O157	3	NA
2009	British Columbia	Petting zoo	O157	13	NA
2010	Manitoba	Fruit compote	O157	40	NA
2012	New Brunswick	Romaine Lettuce	O157	27	NA
2012	Alberta and Ontario	Ground beef	O157	15	NA
2013	British Columbia	Gouda cheese	O157	23	Fatality: 1
2014	Alberta	Bean sprouts and pork	O157	124	NA
2014	Ontario	Apple cider	O157	3	NA

1.7 TRENDS IN THE DETECTION OF STEC IN FOOD AND WATER

Several methods have been described for the detection and isolation of STEC from food and water matrices. However no single step method has been developed with broad specificity that can detect all clinically significant STEC. Most approaches include a multistep process and begins with an enrichment step. The face of STEC diagnostics is constantly evolving due to the changing needs in testing. The most current practices in the field of STEC detection will be discussed below.

1.7.1 CULTURE BASED METHODS

1.7.1.1 Enrichment

The intrinsic challenges associated with the detection of pathogens from food are the complexity of the food matrix, the uneven distribution of the pathogen on the food sample, low number and injured target cells, and the presence of background microflora. An enrichment step is usually incorporated into testing procedures to help resuscitate and amplify the target cells and to dilute the effects of food matrix components, thereby improving the threshold of detection (98). A number of combinations of media and antibiotics have been used to enrich for STEC. Tryptic soy broth (TSB), buffered peptone water (BPW), *E. coli* broth (ECB), EHEC broth (EHECB), and brain heart infusion broth (BHIB) are some of the most commonly used base media to enrich STEC (99). Bile salts are usually added as a supplement to enrichment broths as they inhibit the growth of most Gram positive background bacteria, while Gram negative bacteria are resistant to the effects of bile salts (100). Various antibiotics have also been used to limit the growth of background bacteria. Some of the most commonly used antibiotics include novobiocin, vancomycin, cefsulodin, and cefixime (101). Novobiocin is an aminocoumarin antibiotic that affects cell division and is mostly effective against Gram positive bacteria and some Gram negative bacteria like *E. coli* ¹⁰². However it has been reported that most clinically relevant STEC like

O157:H7 are resistant to novobiocin, and that other serogroups belonging to the “Big six” have varying resistances to novobiocin. As a result lower concentrations of novobiocin are used to supplement enrichment media for non-O157 STEC (103). Studies have shown that the other antibiotics listed are able to reduce background microflora but are not used routinely due to the fact that different non-O157 STEC have varying sensitivities to these antibiotics (101, 103, 104). Another factor to be considered during enrichment is the incubation temperature. Various studies have looked at enrichment temperatures and have found that the isolation of STEC from food matrices is more efficient at 42°C (105, 106), however some protocols recommend a short resuscitation step at 37°C to rescue injured or stressed cells followed by enrichment in selective media overnight at 42°C (107). In most diagnostic labs however an enrichment at 42°C is performed overnight.

1.7.1.2 Differential and selective solid media

Most of the work on the development of differential and selective media has been focussed on the isolation of O157 serotypes, however a handful of studies have been published that have successfully developed methods to isolate some of the clinically significant non-O157 serotypes. The most commonly used solid media for the isolation of STEC will be discussed.

1.7.1.2.1 SMAC and CT-SMAC

The most commonly used differential/ selective media for the detection of O157:H7 is sorbitol MacConkey (SMAC) agar, which is a modification of the original formulation. MacConkey agar is used for the selective isolation of Gram negative bacteria and for the differential isolation of enteric bacilli. It contains, lactose, bile salts, crystal violet, peptone and neutral red (pH indicator) (108). Its differential properties are based on the ability of bacteria to ferment lactose; where enteric bacteria that are able to utilize lactose give rise to red colonies due to a drop in pH; on the

other hand if lactose cannot be used, peptone is utilized instead resulting in clear colonies caused by an increase in pH. MacConkey agar also serves as a selective medium by inhibiting the growth of most Gram positive bacteria by the action of crystal violet and bile salts (109). O157:H7 can be distinguished from most non-pathogenic and non-O157 STEC by its inability to ferment sorbitol. As a result, sorbitol was used to replace lactose in MacConkey agar in order to differentially isolate this serotype (110). Similarly, a study published in 2002 modified SMAC to isolate strains belonging to the O26 serogroup, by replacing sorbitol with rhamnose which cannot be fermented by members of this serogroup (111). Tolerances to tellurite and cefixime have also been exploited to help isolate STEC using the MacConkey base media (CT-SMAC) (106, 111). A recent study suggested that the presence of *eae* genes strongly correlated with tellurite resistance, and as a result the use of CT-SMAC was recommended for the isolation of O157, O26, O103, O111, O121, and O145 serogroups (112). However this medium has been shown to fail to select and differentiate *stx* negative and sorbitol fermenting O157 isolates obtained from patients (113), and therefore it was recommended that all isolates obtained on CT-SMAC should undergo further testing to confirm identity.

1.7.1.2.2 MUG media

Most *E. coli* species produce an enzyme called β -D-glucuronidase that cleaves 4-methylumbelliferyl β -D-glucuronide (MUG) which gives rise to a detectable fluorescent product-methylumbelliferone (114). Thompson *et al* screened a number of *E. coli* strains and found that strains belonging to the O157 serogroup did not produce this enzyme. As a result, MUG media has been used in conjunction with SMAC plates to isolate and identify O157 isolates. In the same study about 11.7% strains belonging to the O157 serogroup were found to be MUG positive and

interestingly were also *stx* negative (115). These results again suggests that screening on MUG has to be followed up with other more specific tests to determine the identity of isolates obtained.

1.7.1.2.3 Commercial CHROMagar STEC and Rainbow agar O157

CHROMagar STEC™ is a proprietary formulation that allows for the detection of most STEC and is thought to exploit tellurite resistance since strains differentially isolated carried the tellurite resistance gene (116). STEC including O26, O111, O121, O145, and O157 appear as mauve colonies on CHROMagar and can be differentiated from other *E. coli* strains. It should be noted however that CHROMagar does not allow differentiation between detected serotypes. Rainbow agar O157 is another commercially available differential media that exploits the differences in the enzymatic profile of the detected strains. It contains chromogenic substrates to both β -glucuronidase and β -galactosidase, and O157 (black colonies, glucuronidase + isolates are purple-blue), O26 (purple), and O111 (violet-gray) can be isolated and differentiated on the same plate based on these characteristics. It has been reported that in some cases the use of Rainbow agar does not help differentiate non-pathogenic *E. coli* from STEC, and supplementation with either tellurite or a combination of tellurite and cefixime or novobiocin to reduce background hindered the growth of target STEC (117, 118, 119). It should be noted that in both instances not all non-O157 STEC of significance can be isolated on a single plate, and in both cases further testing is required to confirm the identity of the isolates either because there is no differentiation between serotypes (as in the case of CHROMagar) or there is ambiguity in differentiating the serotypes.

1.7.2 IMMUNOLOGICAL METHODS

1.7.2.1 Detection of Shiga toxins

Several methods have been developed that allow for the detection of Shiga toxin production via toxin specific antibodies. These methods include: enzyme linked immunosorbent assays (ELISA),

agglutination assays, and colony blot assays (120, 121, 122, 123). All these methods are preceded by an overnight enrichment step in broth containing mitomycin C or polymyxin B. The addition of these compounds to enrichment broth enhances Shiga toxin expression allowing for the production of levels high enough to be detected in these assays (123, 124). There are several commercially available ELISA based assays that utilize antibodies against both Shiga toxin 1 and Shiga toxin 2 in sandwich ELISA formats (Ridascreen verotoxin EIA, Premier EHEC, and ProSpecT), however these assays are limited as not all Shiga toxin 2 variants can be detected by these methods (125, 126). The VTEC-RPLA commercial kit (Oxoid VTEC-RPLA toxin detection kit) on the other hand exploits agglutination to detect STEC in test samples. In this assay latex micro particles coated in antibodies specific to both Shiga toxins are added to test samples in a V-bottom microtiter plate and incubated overnight. A sample is deemed positive when the antibody bound latex particles form lattice structures in the presence of the toxins forming a diffuse layer at the bottom of a V-bottom micro-titre plate. Another method that employs antibodies for the detection of Shiga toxins are colony blots. In this case, Shiga toxins are detected using antibodies specific to the toxins on blots lifted off of primary isolation media supplemented with mitomycin C (123). One of the major limitations of methods that are based on the interaction between Shiga toxin antibodies and the toxins is that most antibodies produced thus far lack specificity to all variants of Shiga toxin 2 (127) . Another consideration is that though these methods include an enrichment stage that enhances toxin production, not all strains produce toxins to the same level, and therefore it is possible for certain strains to express the target toxins at levels below the detection limit of these assays (128). Therefore all serological tests should be followed up by culturing, biochemical and PCR based verification assays.

1.7.2.2 Immunoassays for the detection of STEC cells

Several commercial kits are available that allow for the detection of STEC cells from food matrices. Two methods that have been used with some success will be discussed. The EHEC-Tek Test is one such assay that follows an ELISA format. Polyclonal antibodies against the LPS of the target pathogens are used to coat the wells of ELISA plates. The sample is then applied to the wells, and mAb against outer membrane proteins are used to detect the captured cells. One group reported that the sensitivity of this assay was negatively affected by components like bile salts, however the detection limits were improved when immunocapture was coupled to the ELISA based assay (129). Another test that is used to detect whole cells was the EZ coli test that uses an immunoassay system in a pipette tip format. The tip is packed with filaments that form three section the top being the positive colour gauge, the middle the negative colour gauge, and the bottom is the test element coated with polyclonal antibodies against the target STEC. The sample is applied to the test portion of the tip, and the bound cells are detected using a secondary antibody. When this method was used to detect O157 from meat, it was found that the detection of low levels of target cells were hindered by the presence of high levels of background microflora (130).

1.7.2.3 Capture: Immuno magnetic separation

The concept of immunoaffinity developed soon after superparamagnetic particles were manufactured in the late 1970s. Immunoaffinity in this context involves the immobilization of target specific antibodies on to the surface of the magnetic particles which can then be used to separate the target from suspension by applying a magnetic field. This method is now commonly known as immunomagnetic separation (IMS), and has been used to separate biological materials (131). IMS was then implemented in the detection of food borne pathogens in the early 1990s with sensitivities as high as 10^2 CFU/ ml being reported for *Listeria monocytogenes* from food matrices

(132), and toxin levels as low as 2.5 ng/ ml of *Clostridium perfringens* type A enterotoxin being reported (133). IMS was then commercialized, and has since been used routinely in food testing. The advantage of using IMS for the detection of foodborne pathogens is that it allows for the simultaneous capture and separation of target pathogens from matrix components and background microflora found in enrichment media. This helps to concentrate the target and minimizes the inhibitory effects of these elements in downstream applications like PCR (134). Additionally the use of IMS on enriched samples followed by plating on selective chromogenic plates has been shown to increase the sensitivity of detection by reducing any background flora that may confound results on chromogenic media (135). There are multiple commercial suppliers of automated IMS systems with Dynabeads®, being among the most commonly used. It should be noted that though IMS improves sensitivity, this method could potentially be hindered by non-specific binding of background microflora.

1.7.2.4 Serotyping

The foundations of serotyping of *E. coli* were based on the work done by Dr. Kauffman, where three surface antigens were found to have agglutination properties in the presence of antiserum. The three antigens that were described by Dr. Kauffman were the O, H, and K antigen. In 1966, an antigenic panel was presented, representing different serotypes of *E. coli* (136, 137). Over the years the number of the different antigens added to the scheme have increased and now consists of 173 O antigens and 56 H antigens (5). The diversity in the O and H antigen among serotypes and the antigen's ability to agglutinate in the presence of antiserum provides a unique means by which serotypes can be differentiated. Serological serotyping capitalizes on these features and is based on the agglutination of latex micro particles coated in polyclonal antibodies against the O-

antigen of the lipopolysaccharide and the H –flagellar antigen of the target pathogen (138). Though the production of polyclonal antibodies is time consuming and expensive, multiple commercial kits are available for this purpose and these allow for the rapid identification of serotypes of interest (139, 140). One of the major benefits of serological based serotyping is that it helps type strains that may not be easily be characterized by selective or differential plating, and those that do not harbour typical genetic markers, as in the case of O157 strains that can ferment sorbitol or lack *stx* genes (113, 141). The production of polyclonal antibodies that are specific to their target (O and H antigen) is an integral requirement for serological testing in the identification of clinically relevant STECs. In 2012, a group that raised polyclonal antibodies against the “Big six” were able to detect 100% of their targets, however some of the antibodies exhibited cross reactivity to other non-O157 serotypes, namely antibodies raised against O111, and O45 cross reacted with O26 and antibodies raised against O26 cross reacted with O111 (142). Kits have been available for the typing of O157 serotype strains, however in the recent past commercial suppliers of agglutination slide kits have developed a line of products that are able to type all the relevant non-O157 STEC in North America (Pro-lab Diagnostics, Prolex™ *E. coli* Non-O157 Kit).

1.7.3 POLYMERASE CHAIN REACTION (PCR) FOR DETECTION AND IDENTIFICATION OF STEC

These methods entail the amplification of virulence genes for the identification of the pathogen of interest by either multiplex PCR, real time PCR (qPCR), or RT-PCR (143-145). In the past, most PCR based identification of STEC specifically of the O157 serotype were based on the presence of the Shiga toxin genes and/or the *eae* gene (146). The commercially available Dupont Bax system is based on the detection of these two genes and is used to rapidly screen samples for the presence or absence of STEC. However a major limitation in this approach was the lack of specificity of the *stx* primers to different subtypes of the Shiga toxin genes (147). Additionally not all clinically

significant STEC are LEE positive and therefore lack the *eae* gene (113). As a result, it is possible that methods based on the detection of these genes alone could give rise to false negative results. In order to overcome these limitations, more virulence genes have been added to the panel for detection and include virulence genes like hemolysin (*hlyA*) (148), flagellar protein (*fliC*) (149), O157 specific antigen (*rfbe*) (150), extracellular serine protease (*espP*) (151), and hypothetical autoagglutination protein gene (*saa* – LEE negative) (152). It has been reported that PCR primers have been developed for six of these genes (*stx* 1, *stx*2, *hlyA*, *rfbe*, and *eae*) so that they may be detected simultaneously via multiplex PCR giving rise to well separated bands following electrophoresis (153). This panel was validated using non-O157 STEC and it could be used to differentiate O157 STEC from non-O157 STEC via the presence or absence of the *rfbe* gene. The panel is limited however, by the fact that it does not allow one to differentiate between non-O157 STEC due to differences in the presence of genes observed between strains of the same serotype. The challenge in PCR based detection is to develop a method that will allow for the simultaneous detection and differentiation of all clinically relevant STEC. To that end, two groups have developed methods that target the *wzx* gene (O antigen flippase) of each of the big 6 and O157 serotypes, and allows for the easy differentiation of the serotypes following amplification (154, 155). A lengthy enrichment precedes the PCR step in all the methods described in this section, to ensure that the cell concentration in the volume sampled (1 ml) is sufficient to allow detection of the target organism. Additionally food matrix components have been shown to inhibit PCR amplification of the target potentially leading to false negatives (156, 157).

1.8 METHODS CURRENTLY USED AT THE CFIA

Several methods for the detection, isolation, and identification of food borne pathogens from food and environmental surface samples have been described by Health Canada in the Compendium of Analytical Methods. There are two methods implemented at the Canadian Food Inspection Agency for the detection of STEC from food and water samples, one of which is specific for O157:H7/NM (MFHPB-10) and the other is a newly validated method that allows for the detection of all clinically relevant STEC (MFLP-52). The different steps that are involved in these two procedures will be discussed in brief.

1.8.1 MFHPB-10: ISOLATION OF E. COLI O157:H7/NM FROM FOODS AND ENVIRONMENTAL SURFACE SAMPLES

The first step of this procedure is primary enrichment. A 1 in 10 dilution of the sample is made in modified tryptic soy broth (25 g or 25 ml of sample in 225 ml of broth) containing novobiocin at a final concentration of 20 µg/ml. The suspension is stomached or vortexed to obtain a homogenous mixture, and then incubated at 42°C for 22-24 hrs. Primary enrichment is followed by immunomagnetic separation (IMS), where 1 ml of the enrichment culture is added to 20 µl Dynabead coated with O157 specific antibodies and incubated at room temperature with gentle agitation for 10 mins. The beads are then washed and resuspended in PBS-tween buffer (Sigma, P3563) and plated on two separate differential/ selective media eg: BD CHROMagar and CT-SMAC or CR-SMAC. The plates are then incubated at either 42°C (mHC, and mHC-CT plates) or 35°C (BD CHROMagar and CT-SMAC, and CR-SMAC) for 18-24 hrs. If large well defined colonies are obtained, confirmatory tests can then be performed on the isolated colonies. If the suspect colonies are not well defined or are small (slow growers), the colonies are subcultured onto selective agar media and incubated as described previously to ensure purity. Ten colonies

picked from the plate are subcultured on selective agar and incubated as described above. Colonies that meet “typical” O157 serotype criteria are then subjected to confirmatory tests (API, cellobiose agar) to verify that the suspect colonies belong to the *E. coli* species, agglutination assays are then performed to determine the O serotype of the suspect colonies, and PCR based test are performed to determine whether the suspect colony carries one or both Shiga toxin genes. If however large well defined colonies are not obtained following IMS, a secondary enrichment is performed using 1ml of the primary enrichment culture in 9 ml of EHEC enrichment broth (EEB) at 35°C for 18-24hrs. IMS is then performed with 1 ml of this suspension and plated on differential and selective media, and if colonies are then obtained the identity of the colonies is confirmed as described above. If the secondary enrichment results in no colonies being detected, the sample is deemed negative. Generally the isolation procedure can take between 3-4 days, while confirmatory tests take between 2-3 days, this translates to a 5 day (minimum) turnaround time from when a sample is received and a result is generated.

1.8.2 MFLP-52: ISOLATION AND IDENTIFICATION OF PRIORITY VEROTOXIGENIC ESCHERICHIA COLI (VTEC) IN FOODS

In this procedure a 1 in 10 dilution of the sample is made in non-selective tryptic soy broth (25 g or 25 ml of sample in 225 ml of broth) and stomached for 2 min to obtain a homogenous suspension. A 4 hr resuscitation step at 42°C is performed following which vancomycin and cefsulodin are added to the enrichment media to a final concentration of 10 µg/ml and 3µg/ml respectively. The suspension is then incubated for 20 hrs at 42°C. Following enrichment 1 ml of the culture is tested for the presence of Shiga toxin genes using PCR. If a positive result is obtained, a serial dilution of 1 ml of the culture is made and plated onto selective Rainbow agar and incubated for 16-24 hrs at 35 ± 1°C. In the event that none or too few colonies are obtained on the selective

media lower dilutions of the enrichment culture are plated. However if lower dilutions or the enrichment culture does not yield any colonies, the sample is deemed negative of contamination. Once an adequate number (50-100) of colonies are obtained on the selective media, a portion of 10 large, well defined colonies is streaked onto a grided non selective BHI agar plate. The other portion of the 10 colonies is then pooled and tested for the presence of Shiga toxin genes using PCR. If a negative result is obtained two more pools will be tested as described above. If a positive result is obtained, colonies from each segment of the grided non selective BHI agar are tested for Shiga toxin genes using PCR. Once a STEC is identified two procedures are performed: EHEC multiplex PCR performed with DIG labelled dUTP (targets: *eae*, *hlyA*, *stx1*, *stx2*, *wzx* genes of clinically significant STEC (“Big six” and O157), and an amplification control) and cloth-based hybridization array system (CHAS). Briefly in CHAS, oligonucleotide probes specific to the multiplex targets are cross-linked onto a cloth strip, the DIG labelled product of the multiplex PCR is then used to saturate these strips. Following incubation and washing steps, hybridization of the probes to their targets is detected using anti DIG antibodies. The entire procedure takes about 4-5 days and has only been validated for beef and raw processed food (vegetables and fruit).

1.9 LIMITATIONS OF TESTING METHODS MFLP-52 AND MFHPB-10

1.9.1 PCR INHIBITORS

As mentioned earlier there are inherent obstacles that are associated with pathogen detection in food matrices and some of these obstacles are prevalent in the methods implemented at the CFIA (Compendium of analytical methods, Health Canada). One of the most important assays used in MFLP-52 is the PCR based detection of the Shiga toxin genes. A major limitation of PCR based methods being implemented on enrichment samples is that food matrix components can inhibit PCR reactions. These components include collagen (156), acidic polysaccharides (157), heme and

its metabolic products (158), and humic substances (159). The very first presumptive positive PCR test in MFLP-52 uses a sample from the enrichment culture to determine if further processing is required. However the sample could contain one or more of the inhibitory components mentioned which could give rise to false negatives resulting in missed detection of STEC.

1.9.2 BACKGROUND MICROFLORA

Multiple studies have reported that naturally occurring background microflora could have an antagonistic effect on the growth of STEC especially in meat (160-162). In order to overcome this effect, procedures for enrichment usually include a long enrichment time and the addition of antibiotics. However, a recent study suggested that once the background microflora reached a maximal bacterial density, the growth of O157:H7 was inhibited (161). The same study reported that the growth of STEC inhibiting background microflora in beef was not inhibited by routinely used enrichment media supplemented with antibiotics. IMS is another technique than can be affected by high levels background microflora leading to reduced specificity following capture from enrichment media (163).

1.9.3 LOW SAMPLING VOLUMES

Pathogenic STEC are frequently found in low number within food matrices, and are usually damaged or stressed (98). Enrichment of the samples in selective media for extended periods of time is intended to overcome this obstacle. Reports on the effect of background microflora on the growth of STEC and the varying sensitivities of STEC isolates to the frequently used antibiotics suggest that there is a potential for the target pathogens to not reach high densities following enrichment (101, 103, 104, 160, 161). This issue is further compounded when the volume sampled

following enrichment is low (1 ml) as in the case of IMS, and PCR based detection methods potentially leading to false negatives.

1.9.4 TIME CONSUMING AND TEDIOUS

Both procedures include an overnight enrichment step, and is usually completed in about 4-5 days. Furthermore the MFLP -52 procedure calls for the testing of Shiga toxin genes at three different steps, and screening is performed on differential media leading to the possible selection of non STEC isolates. In the same procedure, cloth strips to be used can only be obtained from a single lab at the CFIA and from a single commercial supplier with no substitutions being allowed. The tedious and time consuming nature of these procedures could lead to contaminated food being in the market for a sufficient amount of time to cause harm before a recall can be mandated. This was evidenced by the case in British Columbia of contaminated beef being recalled only after its best before date had passed late in 2014 (164).

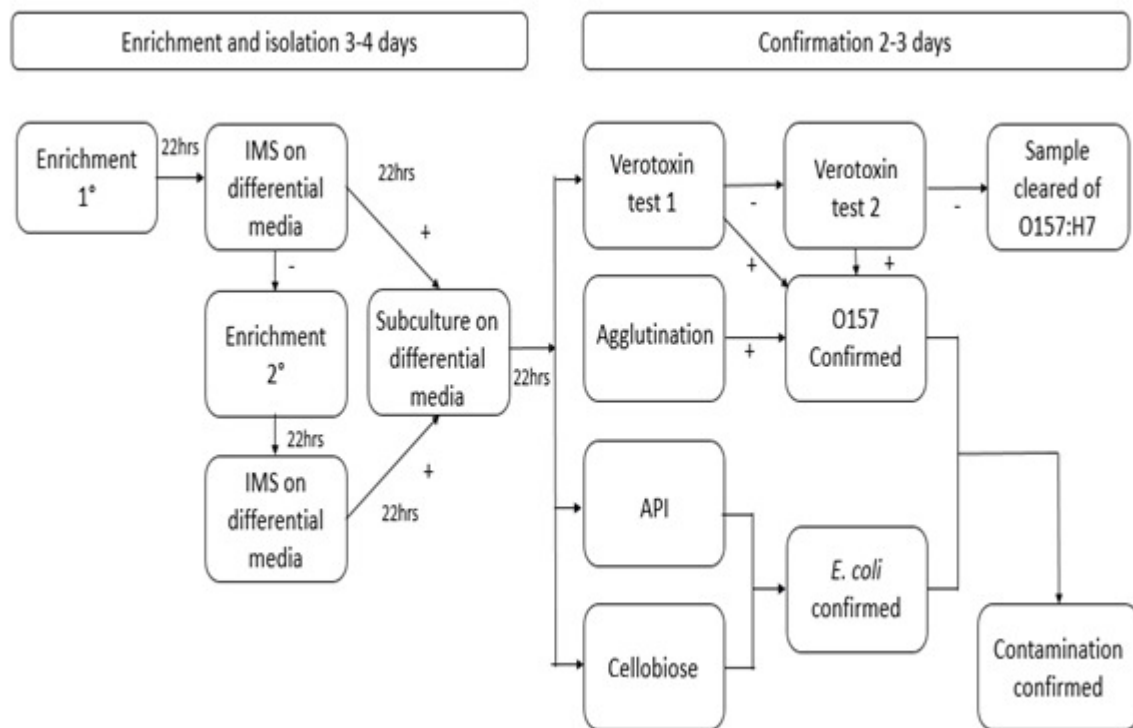


Fig 4: Work flow associated with the MFHPB-10 procedure. Procedure used when testing food, water and environmental samples for O157:H7/NM. The method includes an enrichment step followed by IMS, and plating on selective/ differential media. Identification of typical O157 isolates is performed by PCR based verification of *stx* genes, biochemical tests (API), and serotyping.

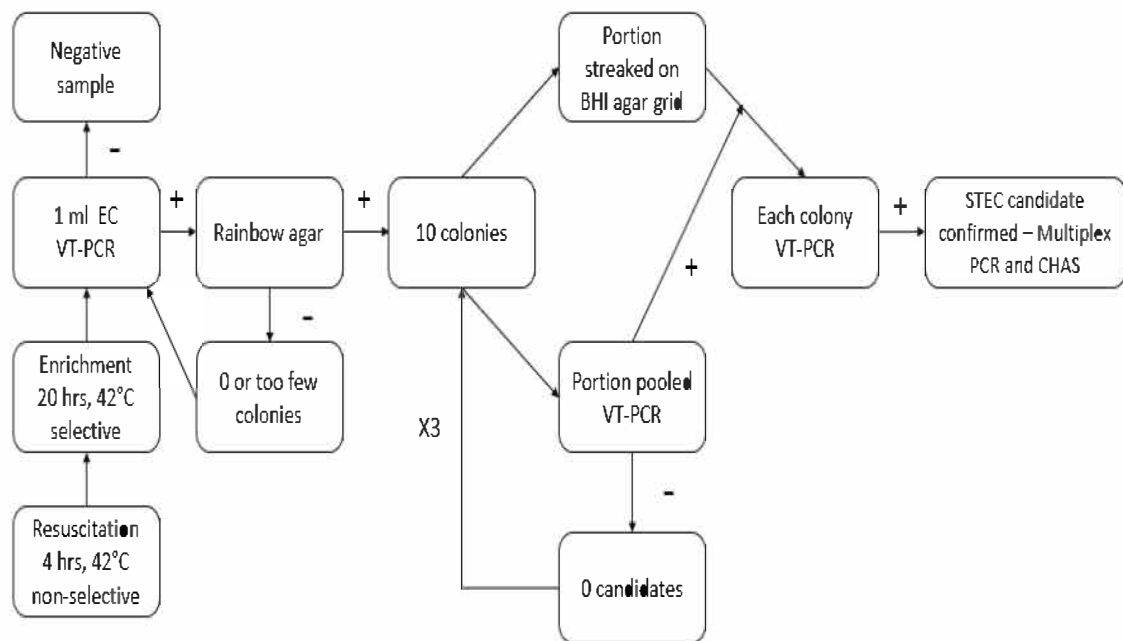


Fig 5: Work flow associated with the MFLP-52 procedure. This procedure is used when testing food for the top 7 EHEC serotypes. It includes an enrichment step followed by PCR based detection of stx genes. The culture is then plated on differential/ selective media and colonies so obtained are screened for the presence of genes (*eae*, *hlyA*, *stx1*, *stx2*, *wzx*) that are specific to target STEC via PCR and CHAS.

1.10 RATIONALE OF STUDY

Given that the currently used methods at the CFIA have apparent limitations, the focus of this study is to streamline the testing procedure for STEC in food and water, by reducing enrichment times and to simultaneously capture, isolate, and detect STEC using highly specific antibodies against these pathogens from the entire enrichment volume. By reducing enrichment times, the growth of background microflora can be limited, thereby minimizing the effects of background bacterial density on the growth of the target. Furthermore, processing the entire enrichment volume will circumvent the need for very high bacterial densities of the target to be obtained since this method would in effect concentrate the target. Using mAb that have been immobilized on a solid matrix to capture STEC, helps to minimize the effects of food matrix components on downstream processes and reduces the contaminating background microflora that confound screening on selective/differential plates.

1.11 PREVIOUS WORK

Multiple initiatives have been undertaken at the CFIA aimed at developing methods to detect clinically significant STEC from food and water. One such program headed by Dr. Brooks (Ottawa Laboratories Fallowfield, CFIA) resulted in the development of mAb that were specific to the LPS O antigen of the seven clinically significant STEC (165). Briefly, formalin killed whole cells of the seven STEC were used to inoculate BALB/c and Swiss Webster mice (Day 0, 14, and 28) and the spleens harvested at day 48. Hybridomas were produced by fusing the Sp 2/0-Ag14 myeloma cells with the spleen cells. Indirect enzyme-linked immunosorbent assays (ELISA) were performed to screen for antibodies produced by the hybridomas using proteinase K digests of the target bacteria as the immobilized antigen. Once candidate hybridomas were identified they were expanded, and the mAb produced were screened to determine that they were reactive to the O

antigen of their respective targets. The antibodies were then isotyped, and their specificity was tested by screening them against multiple isolates from the target serotype, the other 6 STEC serotypes, and non *E. coli* species. The results suggest that the antibodies produced were highly specific to the O antigen of their respective targets, with close to no cross reactivity observed with other serotypes of *E. coli* and other bacterial species. These features make these antibodies valuable tools that can be utilized for the capture and recovery of STEC from food and water matrices. The study herein describes the development of two methodologies that will allow the utilization of the antibodies produced above for the simultaneous capture and recovery of STEC from food and water matrices following a short enrichment phase.

1.12 HYPOTHESIS

The use of monoclonal antibodies against STEC LPS O antigen, immobilized on a solid matrix, will allow for the capture and recovery of these pathogens from the entire enrichment volume, allowing for shorter enrichment times and reducing test turnaround times

1.13 OBJECTIVES

1. To determine if multi stage filtration of the entire enrichment volume coupled with pathogen recovery using magnetic beads coated with mAb could allow for shorter enrichment times for samples of food and water
2. To determine if filtration through a column containing antibody coated beads (glass or polystyrene) would allow for the simultaneous capture and recovery of the pathogens of interest from the entire enrichment volume, thereby reducing enrichment time

2.1 INTRODUCTION

2.1.1 FILTRATION

A crucial step associated with pathogen detection in food matrices, is to concentrate, and isolate the target pathogen from food matrix components. Filtration offers an attractive, cost effective, scalable means to clarify food matrix components from enrichment suspensions. However a drawback of this method is that more complex matrices tend to clog membranes and slow down flow rates. Pre-filtration with larger pore sizes (5 μm membranes and cheesecloth) (166, 167), and the treatment of enrichment suspensions with enzymes (trypsin), and detergents (Triton X) to improve filterability have been reported (168). In most of these studies, very small volumes (~2 ml – 10 ml) of unenriched media were filtered for the detection of the target pathogen. However, this procedure would not be optimal in STEC diagnostics since in most cases food samples are contaminated with very low counts of STEC, and an enrichment step is critical to amplify these pathogens to detectable levels (98). Additionally, though treatment with enzymes and detergents greatly improves filterability, it also adversely affects the viability of cells and therefore hinders any downstream applications that require the growth of the target cells (169).

2.1.2 DIRECT EPIFLUORESCENT TECHNOLOGY (DEFT)

Direct epifluorescent microscopy is a method that is used for the direct enumeration of bacteria on a filter membrane. In the case of food borne pathogens, this step is usually preceded by filtration of a small volume of food matrix suspension in a diluent (saline or media), followed by probing with fluorescently tagged antibodies against the pathogen, and visualization under a fluorescence microscope. This method has been reported to be useful in confirming bacterial contamination of ground beef with low levels of O157 (~3.75 CFU/ 25 g) following enrichment (170). Visualization

of the pathogens of interest in this method is sometimes complicated by the concentration of food matrix components, and other bacteria on the membrane (171). Additionally, the quality of the results obtained is heavily dependent on the quality and specificity of the antibodies being used for detection (168). In most studies that utilized filtration as a means to clarify the matrix suspension, only a small volume of unenriched sample was processed. This could potentially lead to missed detection due to the contamination of food matrices with low numbers of STEC. The approach described in this chapter assessed whether the entire volume (250 ml) of enrichment media can be filtered through a series of membranes with decreasing pore sizes following a brief enrichment period which allows the cells to grow to detectable levels, enabling isolation of the target from the membrane using IMS. The potential to visualize the target captured by IMS was also be determined. This procedure allows for the target to be concentrated from the entire enrichment volume, as a result, high bacterial densities do not need to be attained to meet the requirements for most IMS procedures. mAb raised against the O antigen of LPS of STEC developed by Dr. Brooks have been shown to be extremely specific to their targets (165), and as a result are excellent candidates that can be used in IMS. Visualization via immunofluorescence microscopy following IMS allows for easy visualization of the target pathogens since it separates the target cells from background microflora, and food matrix components.

2.2 EXPERIMENTAL DESIGN

In this section we tested the capture efficiency and specificity of the mAb produced by Dr. Brooks in the presence of a competing organism. Once antibodies were screened, they were used for capture in all successive experiments. Furthermore, the ability of a gradient filtration system (180 μm – 0.45 μm) to gradually eliminate food matrix components from enrichment culture containing a 1 in 10 dilution of the food sample was assessed. Those matrices that were able to be filtered in

this manner were then spiked with low levels of STEC, enriched for a short period of time (4 hrs); the entire enrichment volume was then filtered, and mAb coated Dynal beads were used to recover the target pathogen from the membrane. Similarly the ability of the target STEC to reach detectable levels under the same conditions in the presence of a competitor was assessed. Following recovery, two approaches were pursued: (1) the cells were plated to determine if a short enrichment period was sufficient to capture target STEC via IMS; (2) the ability to visualize eluted pathogen that had been concentrated on a 0.2 μm isopore polycarbonate membrane was assessed with a fluorescence microscope once it had been probed with the appropriate antibody.

2.3 MATERIALS AND METHODS

2.3.1 FILTRATION MEMBRANES

Millipore filtration membranes made from different materials and pore sizes were utilized. The different membranes utilized for gradient filtration include: 180 μm (Nylon Net Filters, Millipore), 100 μm (Nylon Net Filters, Millipore), 41 μm (Nylon Net Filters, Millipore), 10 μm (Nylon Net Filters, Millipore) and 5 μm (Nylon Net Filters, Millipore) net filter membranes (47 mm dia.), while 0.4 μm and 0.2 μm black polycarbonate filter membranes (25 mm dia.) were used for the final isolation and visualization of STEC, respectively.

2.3.2 FOOD MATRICES

Tap water, clear apple juice, medium ground beef, and lettuce were the food matrices tested in this section. Medium ground beef bought at a local butchery was aseptically divided into 25 g portions and each portion was individually wrapped and frozen. Similarly, lettuce leaves procured from a local market were divided into 25 g portions and individually wrapped in saran wrap and stored at

CHAPTER 2: Gradient filtration

4°C for up to a week. Minute Maid pasteurized apple juice and tap water were the liquid matrices tested in this section and was aliquoted into 25 ml portions when required.

2.3.3 MEDIA, BUFFERS, AND BEADS

All overnight pure cultures were prepared in Luria- Bertani (LB) broth. Modified tryptic soy broth (mTSB) was used for all enrichment procedures. Ground beef samples were enriched in mTSB containing 2% Triton X. Protein G coated Dynabeads ® (Thermofisher) were used as the solid substrate for the immobilization of mAb. All dilutions of bacterial cultures, binding of mAb, washes, and the resuspension of beads following capture were performed in PBS (10mM, pH 7.4). Blocking, washes, and antibody dilutions were prepared in PBS containing 0.2% (v/v) Triton X and 0.05% (v/v) Tween 20 (PBS-TT) for colony blots.

2.3.4 COATING DYNAL PROTEIN G MAGNETIC BEADS WITH MONOCLONAL ANTIBODIES

Fifty microliters of Dynal protein G magnetic beads were washed twice with 1 ml of PBS. Two hundred microliters of a 1 in 40 dilution of mAb (tissue culture fluid (TCF) from hybridomas grown in a bioreactor under serum free conditions) specific to the target serotype in PBS was then added to the washed beads. The beads were then incubated for 20 min at room temperature (RT) with end over end mixing. Following incubation the beads were washed 3 times with 1 ml of PBS and the supernatant removed following magnetic separation.

2.3.5 STRAINS AND MONOCLONAL ANTIBODIES

For specificity studies, one isolate per serotype was utilized for capture and colony blotting. Two isolates of each serotype were used in all enrichment studies. mAb specific to each of the target O antigens were provided by Dr. Brooks. The different serotypes and mAb used have been listed in Table 2.

Table 2: List of STEC isolates and the corresponding monoclonal antibodies used for capture experiments using IMS

Serotype / Species	Isolate 1	Isolate 2	Monoclonal antibody
<i>E. coli</i> O157	87-1215	EC1930550-278F	M3475
<i>E. coli</i> O26	02-6737	EC930522	M3354
<i>E. coli</i> O45	05-65545	1980	M3359
<i>E. coli</i> O103	04-2446	EC920185	M3385
<i>E. coli</i> O111	00-4748	EC930489	M3301
<i>E. coli</i> O121	03-2832	-	M3436
<i>E. coli</i> O145	03-4699	001	M3369
<i>Salmonella</i> Typhimurium DT104	SA03-1907	-	M3037
<i>E. coli</i> non pathogenic	ATCC25922	-	-

2.3.6 COMPETITIVE CAPTURE TO DETERMINE SPECIFICITY OF ANTIBODIES

Overnight cultures were prepared for one isolate per serotype tested by adding a single colony from fresh LB agar plates into 5 ml of LB broth. The suspension was then incubated at 37°C with agitation (250 rpm) for 19 hrs. Following incubation, the optical density of the overnight cultures were adjusted to $OD_{600} = 1$ ($\sim 1 \times 10^9$ CFU/ ml). The STEC suspensions were then serially diluted in PBS as required for successive steps. A 1 ml bacterial suspension in PBS containing 1×10^3 CFU of the STEC of interest and 1×10^4 CFU/ ml of either the *Salmonella* Typhimurium or non-pathogenic *E. coli* suspensions was added to the antibody coated beads. The beads were then allowed to incubate at RT for 30 min with end over end mixing. Following incubation, the unbound bacteria were removed following magnetic separation; the beads were then washed 4 times with PBS with the beads being transferred into fresh tubes between each wash. The beads were then resuspended in PBS (500 μ l) and plated on LB agar (100 μ l/ plate).

CHAPTER 2: Gradient filtration

2.3.7 COLONY IMMUNOBLOTTING

Nitrocellulose membranes were cut to fit a standard culture plate and placed over bacterial colonies on LB agar plates obtained from capture experiments. After 30 seconds, the membranes were lifted off of the plates and placed face up in glass petri dishes containing blotting paper cut to size. The membranes were submerged in chloroform for 5 minutes, allowed to air dry, and washed vigorously with PBS-TT to remove any cell debris on the membrane. The membranes were then blocked with PBS-TT containing 3% (w/v) bovine serum albumin (BSA) at 4°C. Following blocking, the membranes were probed for 1 hr at room temperature (RT, 22°C) with either a 1 in 100 dilution of the mAb in PBS-TT against the STEC of interest or a 1 in 25 dilution of mAb against *Salmonella* Typhimurium. The membranes were then washed 3 times for 5 minutes with PBS-TT and probed at RT with a 1 in 2000 dilution of horse radish peroxidase conjugated goat anti mouse antibody for 1 hr. The membranes were then washed 5 times with PBS-TT for 5 minutes, and were developed for 10 minutes (Bio-Rad HRP conjugate substrate). Development was stopped by washing the membranes in double distilled water and images were taken after the membranes were allowed to air dry.

2.3.8 FILTRATION POTENTIAL OF DIFFERENT FOOD MATRICES

A 1 in 10 dilution of the different food matrices were made in mTSB in a final volume of 250 ml (25 g of solid matrices or 25 ml of liquid matrices in 225 ml of mTSB). All diluted solid matrices were placed into filtered stomacher bags and homogenized for 2 min in a stomacher. All homogenous media suspensions were subjected to gradient filtration. Briefly, the filtrate from one filtration procedure was used as the feed solution for the next filtration procedure, with the pore size of the filtration membrane decreasing sequentially. In this manner the ability to filter the diluted matrix suspension through 180 µm down to 0.4 µm filters was tested.

2.3.9 ENRICHMENT AND CAPTURE OF STEC FROM SPIKED APPLE JUICE AND WATER MATRICES

Overnight cultures were prepared by adding a single colony from fresh LB plates into 5 ml of LB broth. The suspension was then incubated at 37°C with agitation (250 rpm) for 19 hrs. Following incubation, the optical density of the overnight cultures were adjusted to $OD_{600} = 1$ (1×10^9 CFU/ml). The STEC suspensions were then serially diluted in PBS as required for successive steps. Twenty five ml of tap water or clear apple juice were spiked with 1-15 CFU (confirmed by plating the bacterial suspension corresponding to 10^3 CFU/ml on LB agar) of the STEC of interest and then added to 225 ml of mTSB media. The suspensions were then incubated with agitation (250 rpm) for 4 hrs at 37C. Following incubation the suspension was filtered through a 0.4 μ m isopore filter using a pump (~12 in Hg). The membrane was then washed with PBS, gently removed from the filtration apparatus and placed in 2 ml of PBS to recover pathogens from the membrane by vortexing in three short bursts (10 seconds). The supernatants were then added to mAb coated Dynal protein G beads and incubated at room temperature for 30 min. Following incubation, the beads were washed 4 times in PBS and the beads suspended in PBS. The resuspended beads were then plated on non-selective LB agar. In order to ensure that all captured organisms were not endogenously found in the matrices tested, capture experiments were performed with unspiked water and juice.

2.3.10 GROWTH AND CAPTURE OF STEC UNDER ENRICHMENT CONDITIONS IN THE PRESENCE OF A COMPETITOR

Water and juice matrices were spiked with 1-15 CFU of the pathogen of interest and 10-15 CFU of a competitor organism – *Salmonella* Typhimurium or non-pathogenic *E. coli*. The rest of procedure was performed exactly as described in the previous section.

2.3.11 IMMUNOFLUORESCENCE MICROSCOPY

Target STEC were used to spike tap water and then enriched as described above. Following enrichment the suspension was filtered through 0.4 µm isopore polycarbonate membranes. The membranes were then removed and placed in 2 ml of PBS and vortexed in three short bursts to dislodge the bacteria from the membrane. The supernatant was then added to mAb coated Dynal protein G beads. The captured bacteria were then eluted using 1 ml of IgG elution buffer (Thermoscientific) collected in 200 µl fractions. The pooled eluent was then neutralized using 1M Tris (pH 9) and was then filtered through a 0.2 µm isopore polycarbonate membrane and the membranes were blocked with 0.6% (w/v) BSA (IgG free) in PBS for 30 min. The membranes were then probed with a 1 in 200 dilution of the mAb in PBS containing 0.6% (w/v) BSA specific to the target for 30 min at room temperature. The membranes were then washed with 10 ml of PBS and probed with a 1 in 200 dilution of Alexa Fluor ®488 – conjugated goat anti–mouse in PBS containing 0.6% (w/v) BSA and incubated in the dark for 30 min at room temperature. The membranes were then washed with 10 ml of PBS, and transferred from the filtration apparatus onto a slide, and visualized using fluorescence microscopy. As a control 250 ml of unspiked media was treated in the same manner as the spiked media for immunofluorescence microscopy, with the exception that the primary antibody consisted of 1 in 200 dilution of the mAb against all 5 serotypes tested.

2.4 RESULTS

2.4.1 MONOCLONAL ANTIBODIES ARE SPECIFIC TO THEIR TARGET STEC

Capture studies performed using mAb coated Dynal beads indicated that the antibodies were able to bind their target specifically in the presence of an excess of a competing species. Colony blot analysis of the bacteria captured showed that for serotypes O157, O26, O45, O103, O111, O121,

and O145, mAb were able to specifically bind to their targets in the presence of non-pathogenic *E. coli* with a negligible amount of the competitor being captured (≤ 10 CFU) (Fig 6A, please refer to Appendix A1.1-A1.2 for images of colony blots resulting from specificity studies for other serotypes). However, colony blots resulting from capture studies using *Salmonella* Typhimurium as a competitor indicate that the mAb against O121 bound to the competitor non-specifically under the capture conditions used (≥ 20 CFU/ plate) (Fig 7A). However, minimal cross reactivity was observed between the other mAb against the target STEC and the isolate of *Salmonella* Typhimurium during capture (Fig A1.3-A1.4). As a result it was decided that the antibodies against O121 would not be used in further capture experiments. Blots resulting from control plates containing either non-pathogenic *E. coli* or *Salmonella* Typhimurium, and probed with mAb against the target STEC indicated that there was no cross reactivity between the mAb and the competitors (Fig 6B, 7B, refer to Appendix A1.5-A1.8 for colony blots of other serotypes). Furthermore, blots resulting from control plates containing the target STEC or *Salmonella* Typhimurium revealed that the mAb against target STEC and *Salmonella* Typhimurium were specific to their target (Fig 8. A1.9-A1.10).

2.4.2 FILTRATION POTENTIAL OF FOOD MATRIX

Four food matrices diluted in enrichment media were tested for their ability to be filtered through a gradient of membranes. Simpler matrices which include water and clear juice were easily filtered through the gradient (pore sizes from 180 μm down to 0.4 μm); however, more complex food matrices like lettuce and ground beef were not filterable beyond 5 μm and 41 μm respectively (Table 3). As a result, enrichment, filtration and capture experiments were performed with water and clear juice alone.

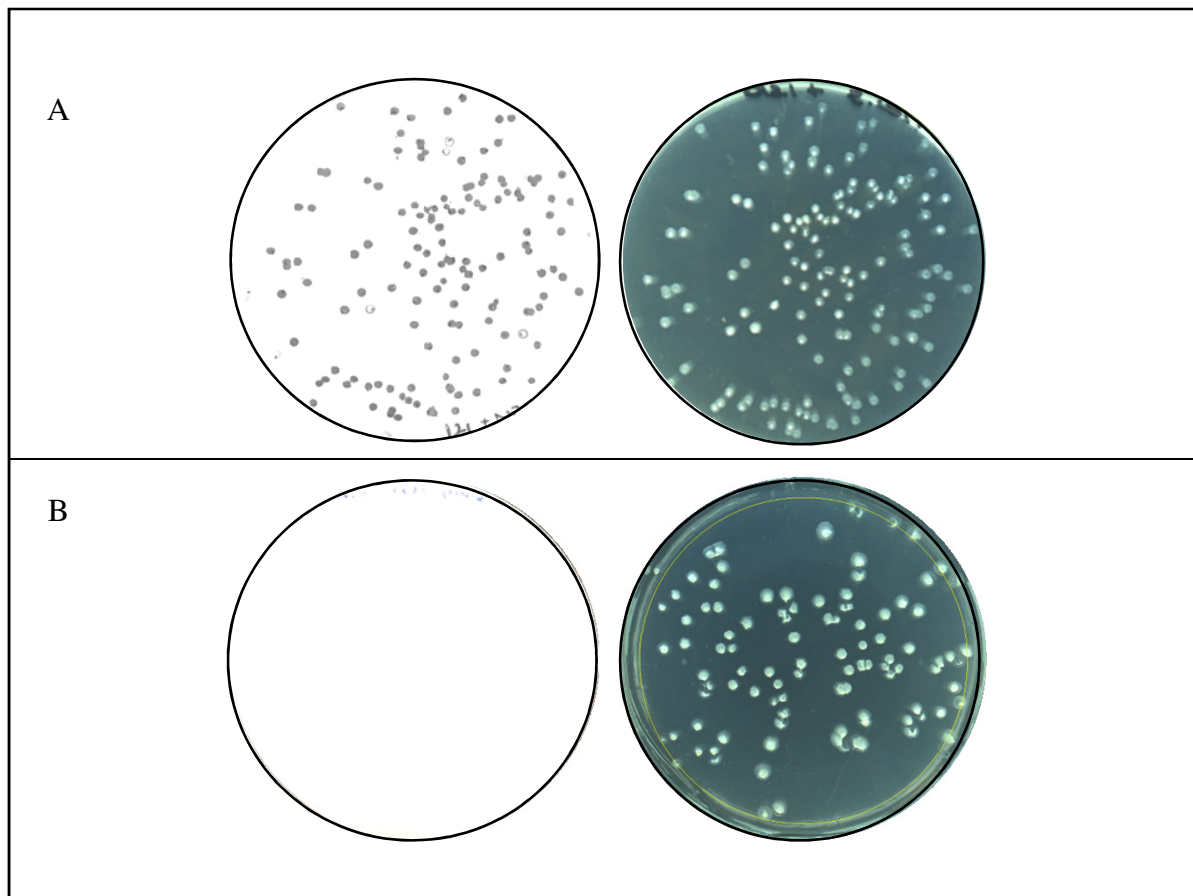


Fig 6: Colony blots resulting from specificity capture studies in the presence of non-pathogenic *E. coli*. Colony immunoblot assays obtained for serotype O121 in the presence of an excess of an isolate of non-pathogenic *E. coli* as a competitor. Capture was performed using Dynabeads® coated with mAb against the O antigen of O121. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. (A) Membrane was probed with mAb specific to the O antigen of serotype O121. (B) Membrane of a control plate containing only non-pathogenic *E. coli* probed with mAb specific to the O antigen of serotype O121

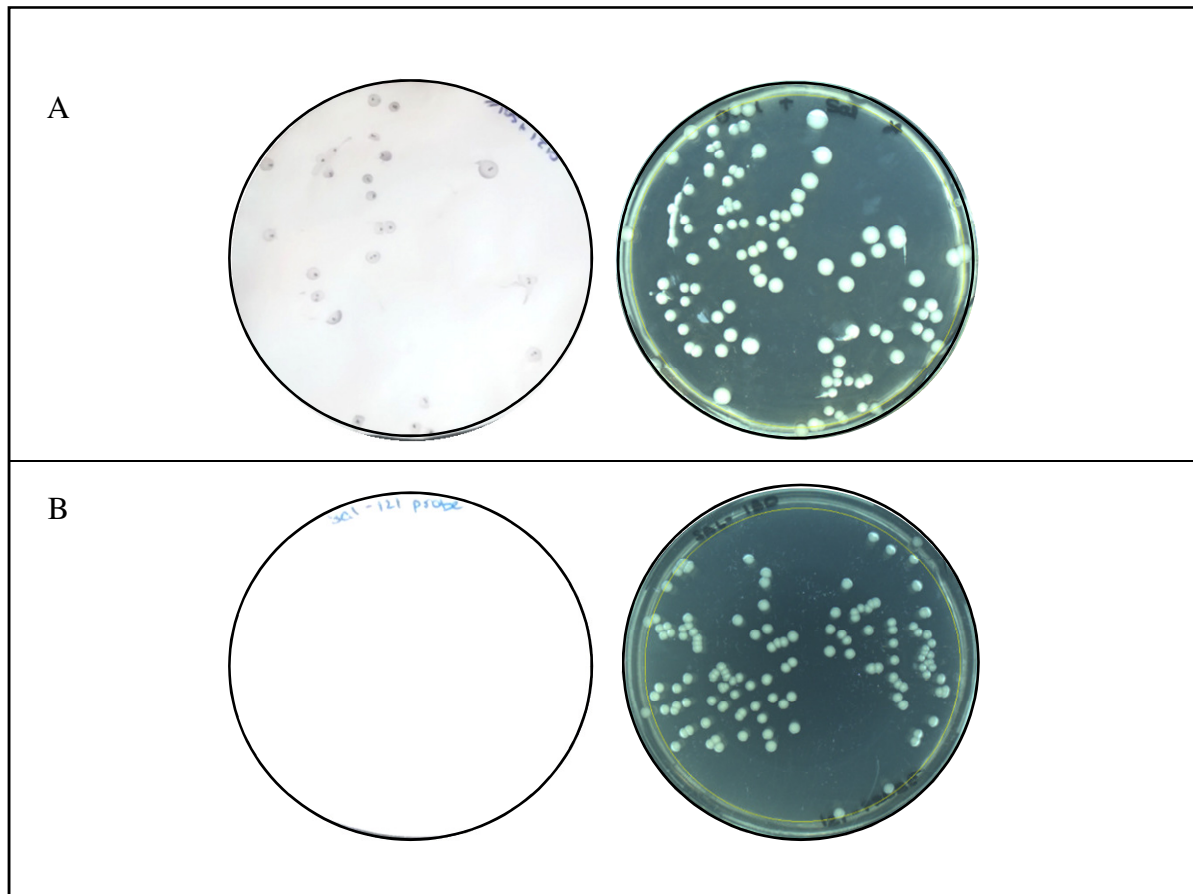


Fig 7: Colony blots resulting from specificity capture studies in the presence of *Salmonella* Typhimurium. Colony immunoblot assays obtained for serotype O121 in the presence of an excess of an isolate of *Salmonella* Typhimurium as a competitor. Capture was performed using Dynal beads coated with mAb against the O antigen of serotype O121. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. (A) Membrane was probed with mAb specific to *Salmonella* Typhimurium. (B) Membrane of a control plate containing only *Salmonella* Typhimurium probed with mAb specific to the O antigen of serotype O121.

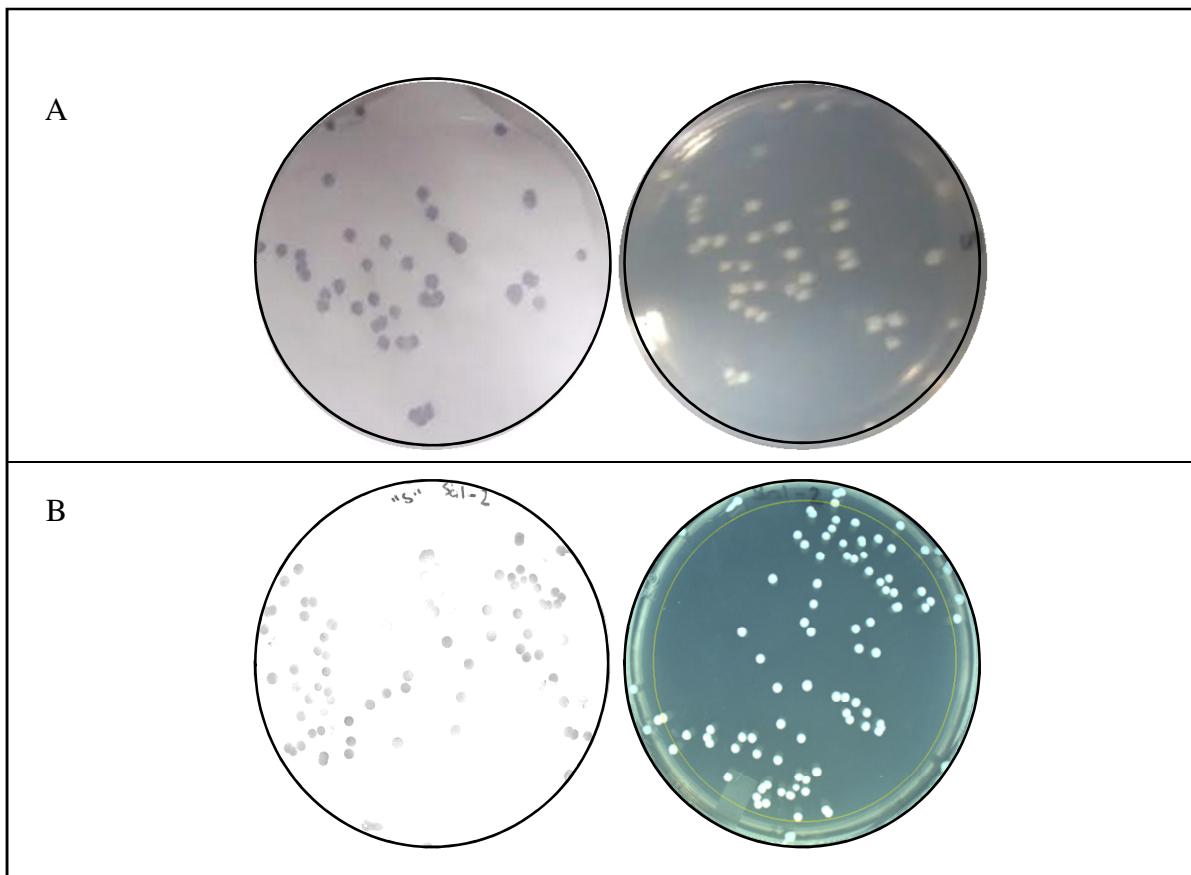


Fig 8: Colony blots of control plates. (A) Membrane from a plate containing only isolates of O121 was probed with mAb specific to serotype O121. (B) Membrane plate containing only *Salmonella* Typhimurium probed with mAb specific to *Salmonella* Typhimurium

Table 3: Filtration potential of various food matrices diluted in enrichment media (1 in 10, 250 ml total volume) through a gradient of filtration membranes

Matrix	180 μ m	100 μ m	41 μ m	10 μ m	5 μ m	0.4 μ m
Water	✓	✓	✓	✓	✓	✓
Juice	✓	✓	✓	✓	✓	✓
Lettuce	✓	✓	✓	✓	✓	x
Beef	✓	✓	✓	x	x	x

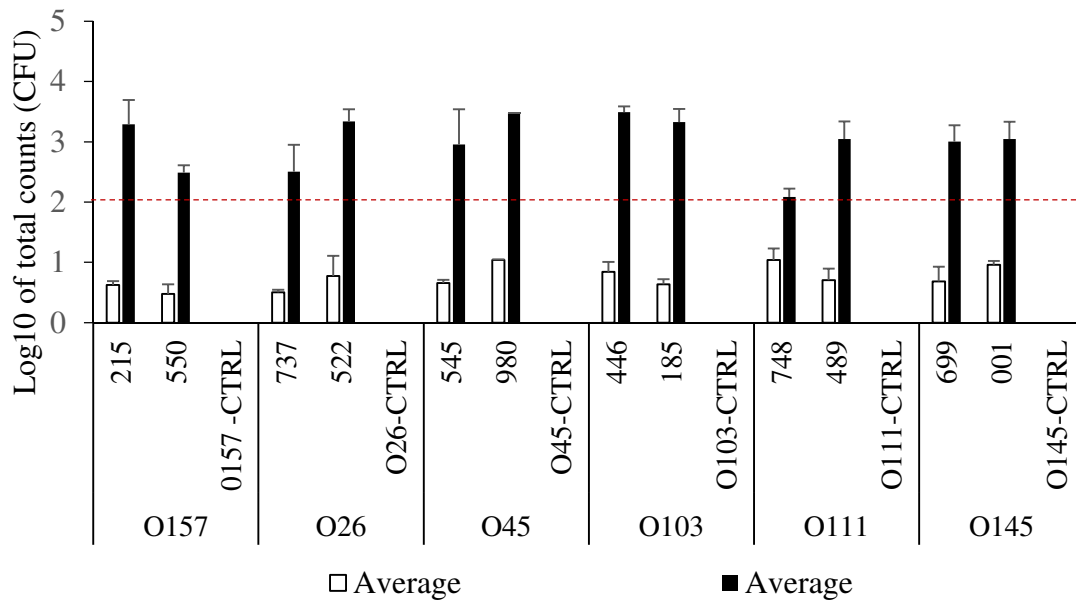
2.4.3 ENRICHMENT AND CAPTURE OF STEC FROM FOOD MATRICES

Two isolates per serotype were used to spike water and apple juice, which were then enriched for 4 hrs. STEC capture experiments performed following filtration indicated that at least 100 CFU of the target STEC could be recovered using IMS (Fig 9). In most cases, counts to the order of 10^3 CFU were obtained with an initial inoculum of ~ 10 CFU within the 4 hr enrichment period. The rate at which the STEC grew was found to be isolate dependent. Isolates belonging to serotype O26 and O111 required a higher initial inoculum ($10 \leq x \leq 15$ CFU) to be able to capture 100 CFU following a 4 hr enrichment. Whereas isolates belonging to serotypes O103 and O145 were able to reach counts to the order of 10^3 CFU or more when inoculated with low initial counts (≤ 10 CFU). No major differences were observed in the capture of STEC when either of the two food matrices were used (apple juice or water).

2.4.4 ENRICHMENT AND CAPTURE OF STEC FROM FOOD MATRICES IN THE PRESENCE OF A COMPETITOR

One isolate per serotype was tested for their ability to reach detectable levels under enrichment conditions in the presence of a competing species inoculated at approximately a 1:1 ratio with the target (~ 10 CFU). As observed in the enrichment study performed earlier isolates belonging to serotypes O26 and O111 required slightly higher inoculation titres to enable the detection of 100 CFU via IMS within the 4 hr enrichment period. The presence and growth of the competitor did not significantly enhance or abrogate the growth of the target STEC when bacteria were enriched with water or juice samples. All isolates could be recovered (at least 100 CFU) using IMS following filtration within the 4 hr enrichment period (Fig 10-12).

A



B

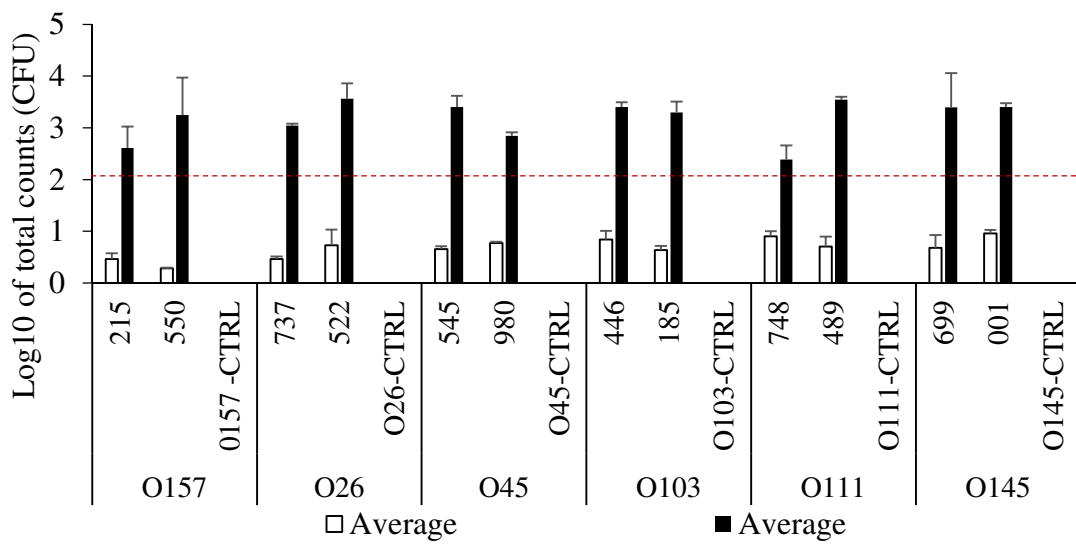
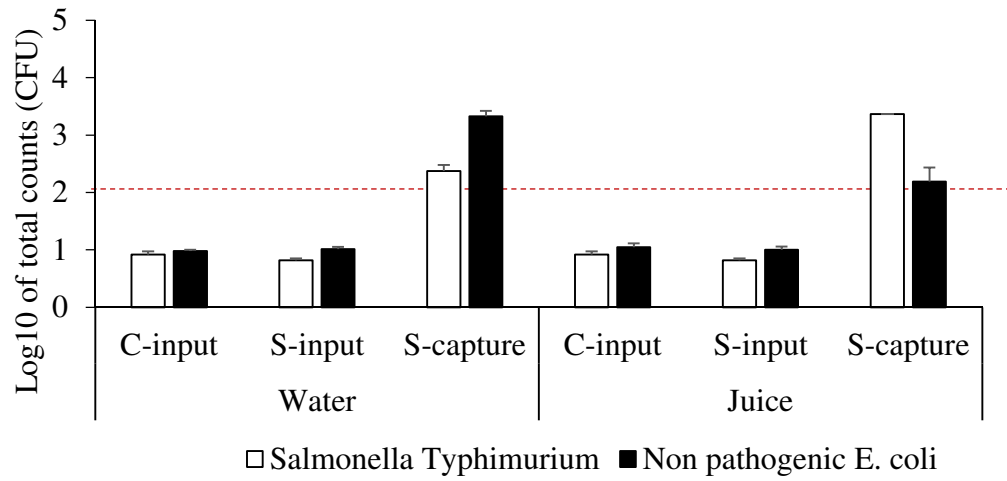


Fig 9: Total counts of the target STEC captured following a 4 hr enrichment. Capture using mAb coated Dynabeads® following the 4 hr enrichment of a water (A) or juice (B) sample spiked with 1-15 CFU of the target in mTSB (n=3). Two isolates per serogroup were tested and the isolate IDs are indicated under each serogroup. Counts obtained from capture experiments of unspiked food matrix enriched for 4 hrs served as a negative control. The mAb coated beads specific to each serogroup were able to capture at least 100 CFU of their target following the short enrichment phase (4 hrs) and is indicated with the dashed line.

A



B

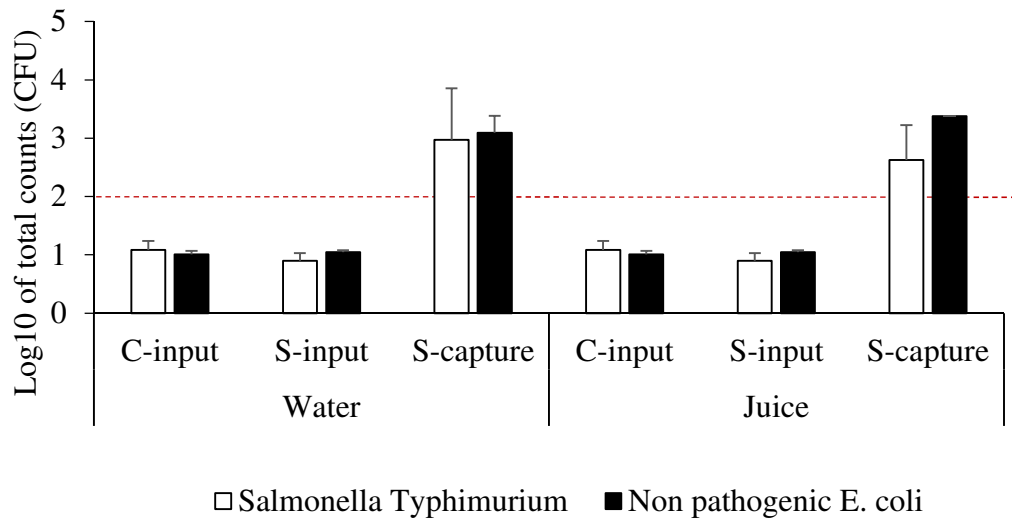
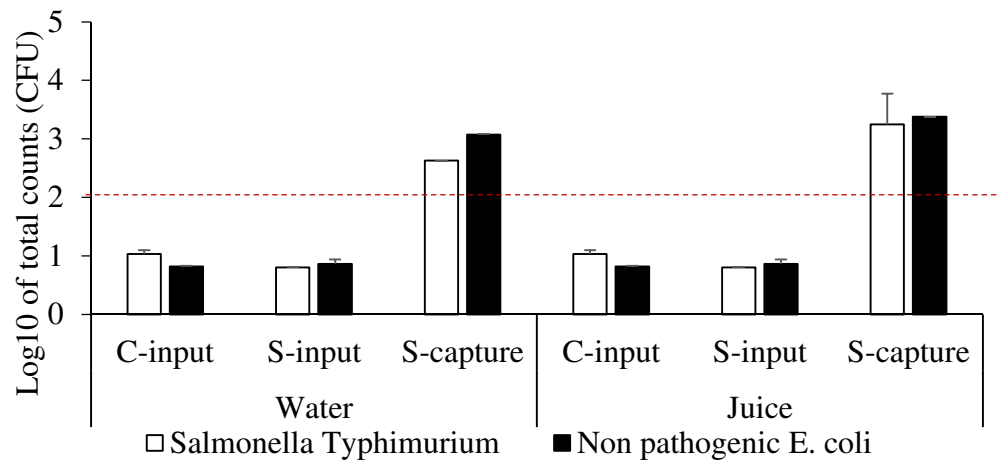


Fig 10: Total counts of the STEC serotypes O157 and O26 captured following 4 hrs of enrichment in the presence of competitors. Capture was performed using mAb coated Dynabeads® following a 4 hr enrichment of a water or juice sample spiked with 1-15 CFU of the target in mTSB, in the presence of an isolate of either *Salmonella* Typhimurium (white bars) or non-pathogenic *E. coli* (black bars) spiked to the same count as the target (n=2). (A) Represents the total counts of an isolate of serotype O157 captured, while (B) represents the counts of an isolate of serotype O26 captured. The mAb coated Dynabeads® were able to capture at least 100 CFU of their target following the short enrichment phase (4 hrs) indicated with the dashed line. The abbreviation used in the graphs are defined as follows: C-input is the total count of the competitor used to spike the matrix, S-input is the total count of the target STEC used to spike the matrix, and S-capture is the total count of the target captured following filtration and IMS.

A



B

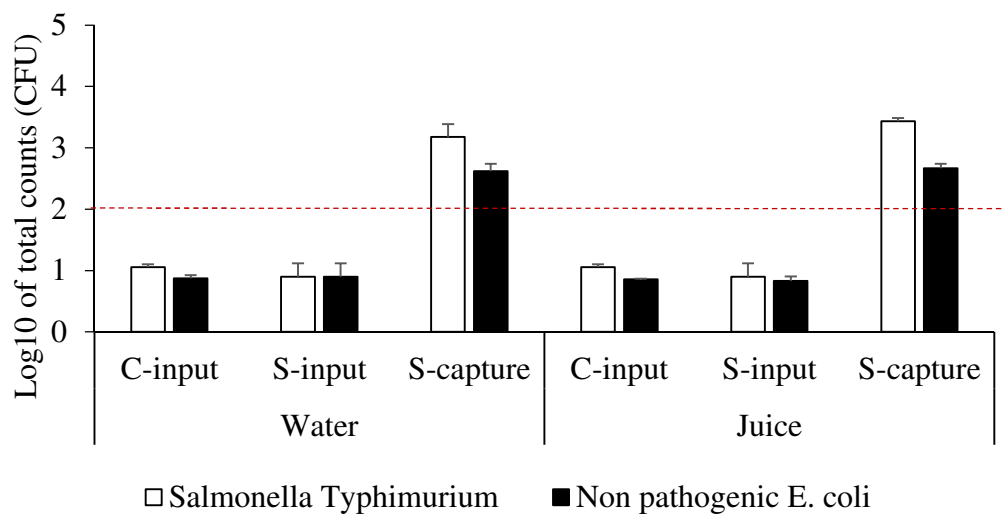
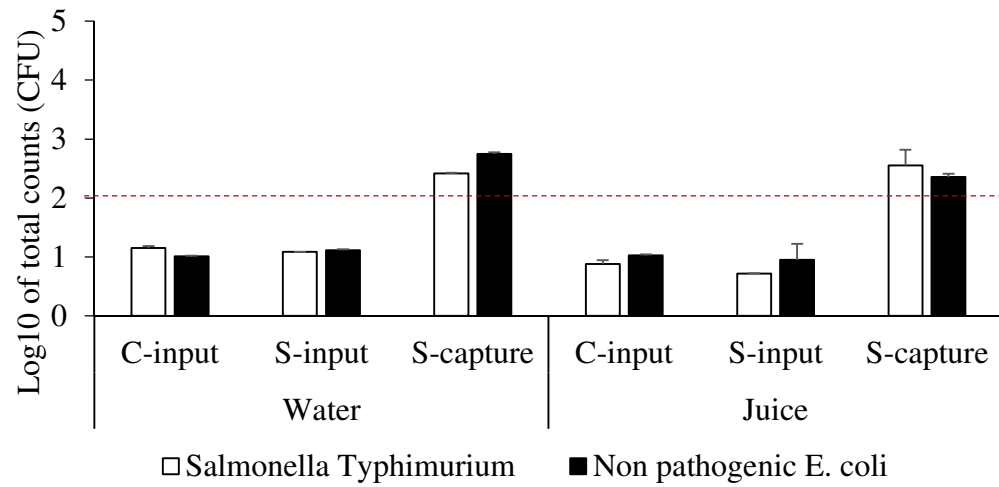


Fig 11: Total counts of the STEC serotypes O45 and O103 captured following 4 hrs of enrichment in the presence of competitors Capture was performed using mAb coated Dynabeads® following a 4 hr enrichment of a water or juice sample spiked with 1-15 CFU of the target in mTSB, in the presence of an isolate of either *Salmonella* Typhimurium (white bars) or non-pathogenic *E. coli* (black bars) spiked to the same count as the target (n=2). (A) Represents the total counts of an isolate of serotype O45 captured, while (B) represents the counts of an isolate of serotype O103 captured. The mAb coated Dynabeads® were able to capture at least 100 CFU of their target following the short enrichment phase (4 hrs) indicated with the dashed line. The abbreviation used in the graphs are defined as follows: C-input is the total count of the competitor used to spike the matrix, S-input is the total count of the target STEC used to spike the matrix, and S-capture is the total count of the target captured following filtration and IMS.

A



B

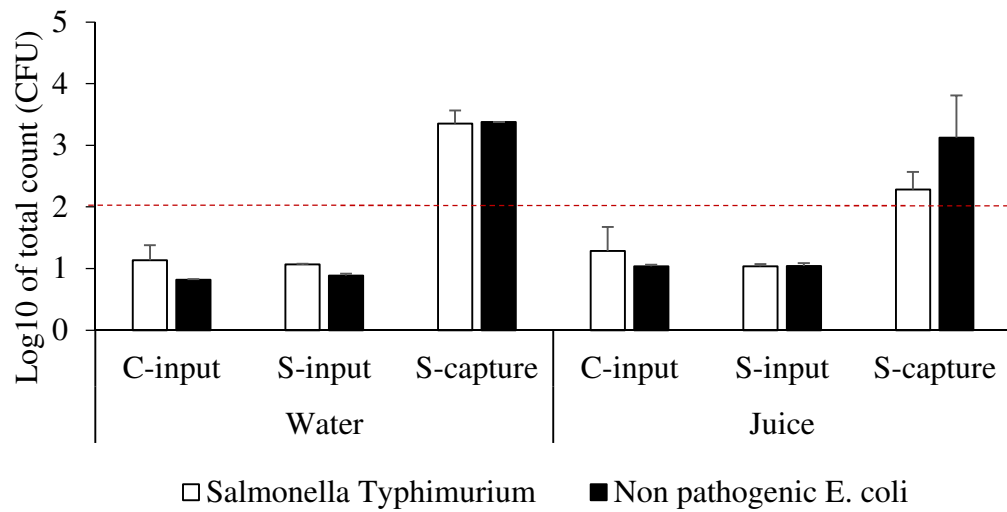


Fig 12: Total counts of the STEC serotypes O111 and O145 captured following 4 hrs of enrichment in the presence of competitors Capture was performed using mAb coated Dynabeads® following a 4 hr enrichment of a water or juice sample spiked with 1-15 CFU of the target in mTSB, in the presence of an isolate of either *Salmonella* Typhimurium (white bars) or non-pathogenic *E. coli* (black bars) spiked to the same count as the target (n=2). Panel A represents the total counts of an isolate of serotype O111 captured, while panel B represents the counts of an isolate of serotype O145 captured. The mAb coated Dynabeads® were able to capture at least 100 CFU of their target following the short enrichment phase (4 hrs) indicated with the dashed line. The abbreviation used in the graphs are defined as follows: C-input is the total count of the competitor used to spike the matrix, S-input is the total count of the target STEC used to spike the matrix, and S-capture is the total count of the target captured following filtration and IMS

2.4.5 COMPETITIVE CAPTURE COLONY BLOTS

Colony blots were carried out on plates resulting from the enrichment, filtration and capture experiments performed in the presence of a competitor. The blots served as a means to confirm that the captured organisms were the target STEC. In experiments where non-pathogenic *E. coli* were used as a competitor, the results indicated that in all cases the bacteria captured by IMS consisted mainly of the STEC of interest (Fig 14, please refer to appendix A2.3-A2.4 for colony blots for other serotypes). Similar results were obtained when *Salmonella* Typhimurium was used as a competitor (Fig 13, please refer to appendix A2.1-2.2 for colony blots for other serotypes).

2.4.6 DIRECT EPIFLUORESCENCE MICROSCOPY

Direct epifluorescence microscopy was performed as an alternative to direct plating following enrichment of spiked water samples. All STEC targets were easily visualized at 40 X magnification using this method, including slow growing serotypes. The negative control did not show any fluorescence on the membrane when probed with mAb against all the target STEC (Fig 15).

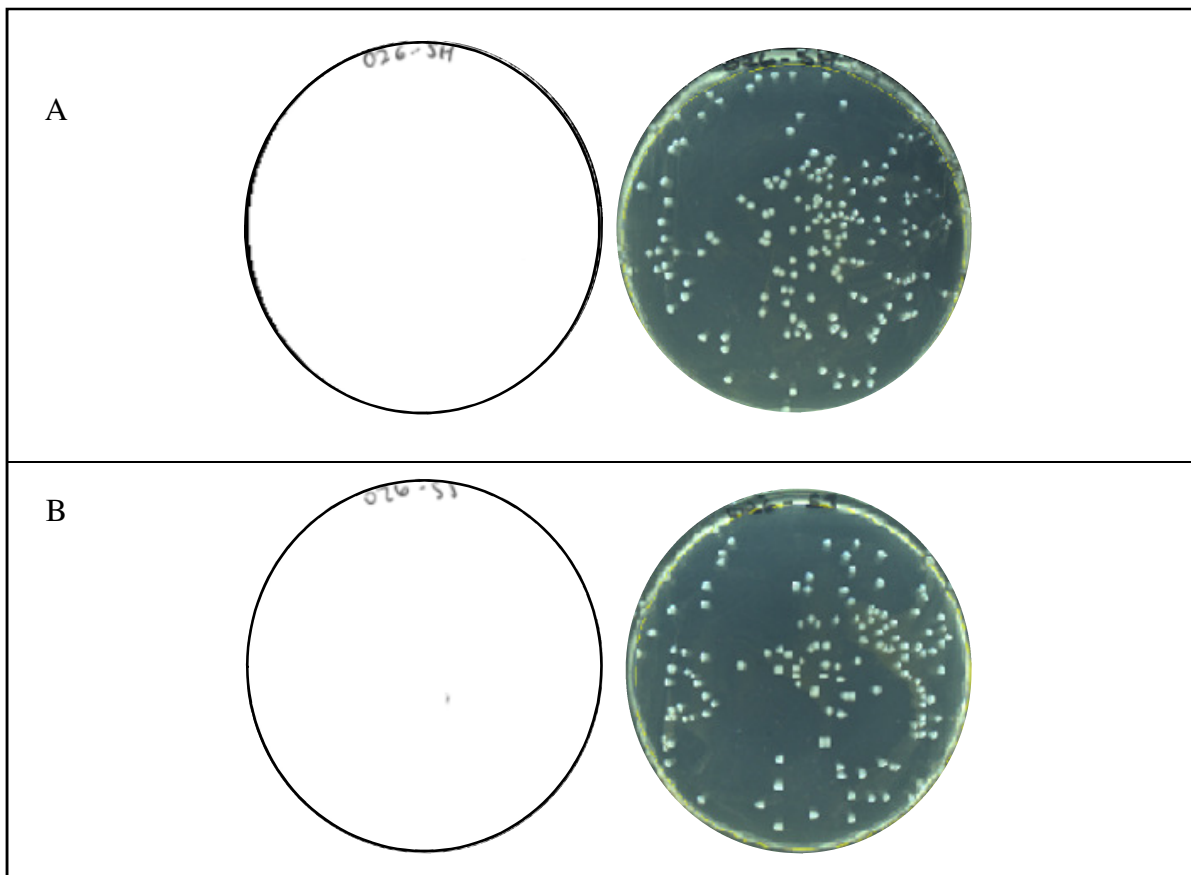


Fig 13: Colony blots resulting from capture of serotype O26 following a 4 hr enrichment in the presence of *Salmonella* Typhimurium. Capture was performed following enrichment of (A) a water sample and (B) an apple juice sample spiked with both an isolate of O26 and an isolate of *Salmonella* Typhimurium as a competitor. Capture was performed using Dynabeads® coated with mAb against the LPS O antigen of O26.

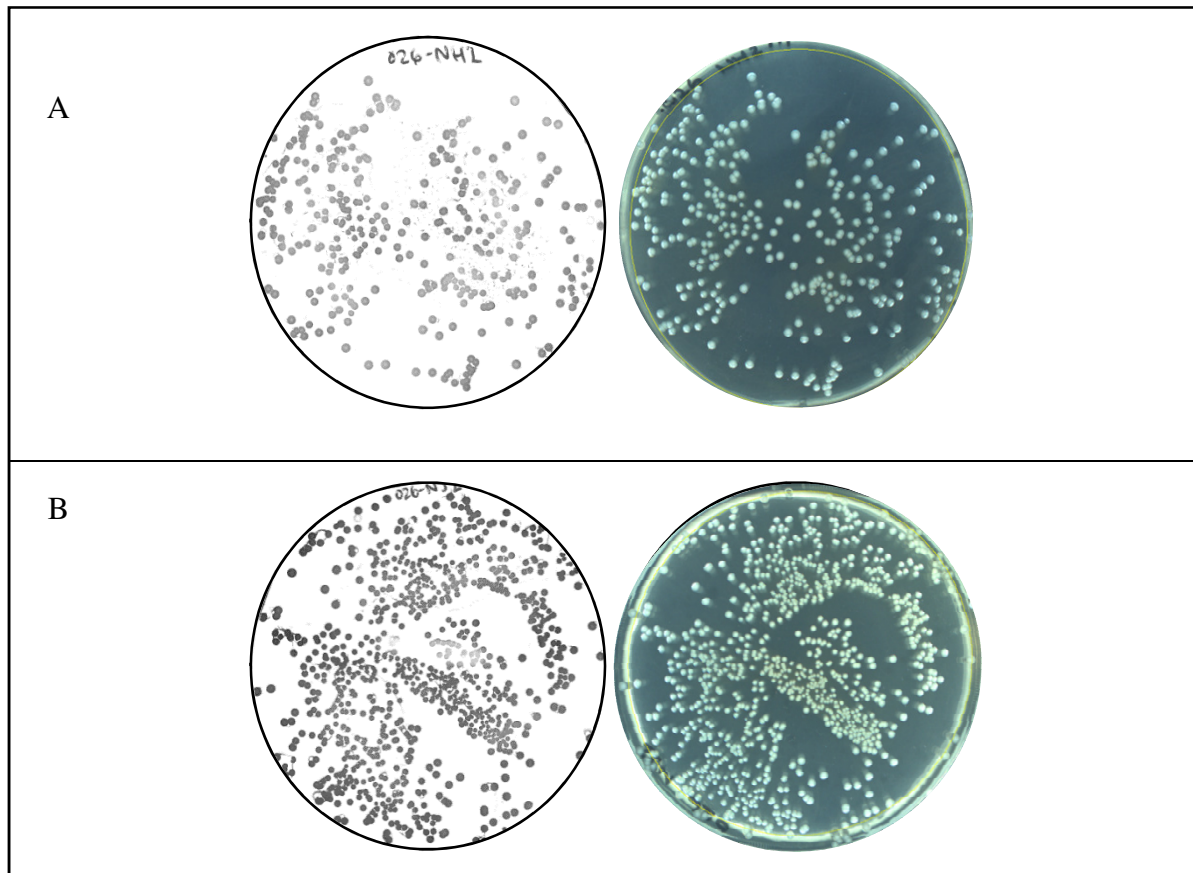


Fig 14: Colony blots resulting from capture of serotype O26 following a 4 hr enrichment in the presence of non-pathogenic *E. coli* as a competitor. Capture was performed following enrichment of (A) a water sample and (B) an apple juice sample spiked with both an isolate of O26 and an isolate of non-pathogenic *E. coli* as a competitor. Capture was performed using Dynabeads® coated with mAb against the LPS O antigen of O26.

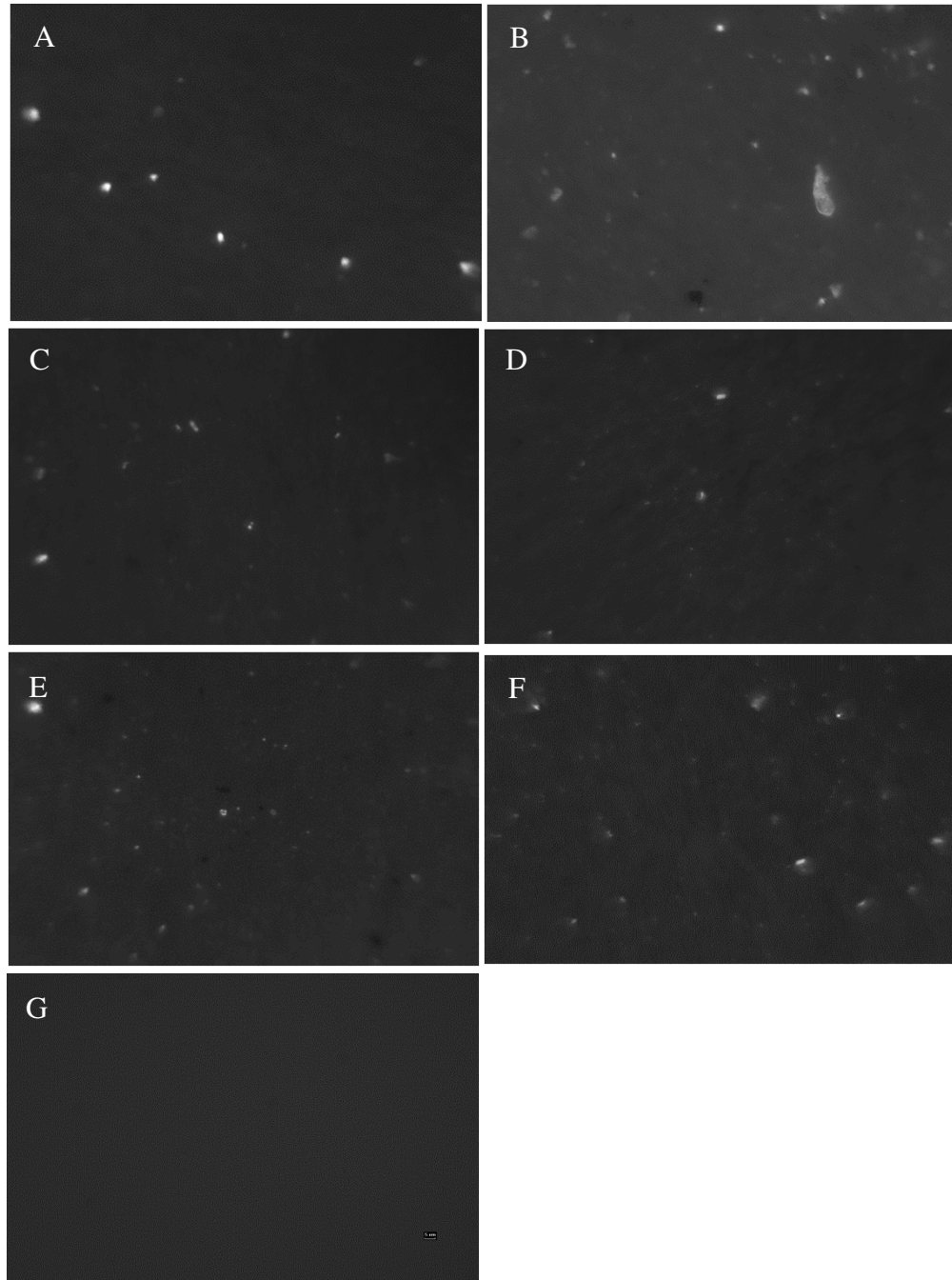


Fig 15: Immunofluorescence microscopy images. IFM was performed following the enrichment of water samples spiked with ~10 CFU of the target STEC for 4 hrs. Target STEC were captured off of 0.4 μ m polycarbonate membranes using mAb coated Dynabeads®. The eluted and neutralized target cells were then filtered through a 0.2 μ m isopore black polycarbonate membrane, and probed with the mAb specific to the target of interest. Panels A-F represent the images obtained for isolates belonging to serogroup O157, O26, O45, O103, O111, and O145 at 40 X magnification. Panel G represents the negative control where an un-spiked water sample was enriched, filtered, and probed with antibodies specific to all 6 serogroups being tested.

2.5 DISCUSSION

The purpose of the work described in this chapter was to develop a method that combines filtration with IMS to recover target bacteria from the entire volume of enrichment culture of food and water samples spiked with a low bacterial load (< 15 CFU), and grown for a short period of 4 hrs. This was achieved by capture of the target by IMS using mAb raised against the O antigen of STEC (serotypes O157, O26, O45, O103, O111, O121, and O145) following filtration through a membrane. The first step in the approach presented here was to determine if the mAb raised against the O antigen of the STEC could capture the target in the presence of a competitor. Preliminary capture experiments performed in the presence of an excess of either *Salmonella* Typhimurium or non-pathogenic *E. coli* (10^4 CFU) indicated that IMS performed with these antibodies allow for the specific and efficient capture of the target STEC (Fig 6-8, A1.1-A1.4). However, when the mAb raised against the LPS O antigen of serotype O121 was used in IMS, non-specific capture of *Salmonella* Typhimurium was observed (Fig 7A) though there was no indication of cross reactivity on colony blots obtained from *Salmonella* Typhimurium plates probed with mAb against O121 (Fig 7B). These results could be due to non-specific interactions between the antibody and the competitor at high concentrations or due to aggregation of the target STEC with the competitor under capture conditions. As a result, the mAb against O121 was not used in any of the enrichment and capture experiments performed. Four different food matrices were tested namely: ground beef, lettuce, water, and apple juice. Filtration of these diluted food matrices in mTSB broth (1 in 10) through a series of membranes with decreasing pore sizes suggested that though this method allows for the gradual removal of food matrix components, the clarification of the suspension was not sufficient to allow for the complete filtration of media derived from the enrichment of beef and lettuce samples (Table 3). This is in agreement with literature that suggests that filtration is mostly beneficial when pathogens are to be separated from

food matrix suspensions that contain low amounts of particulate matter in suspension (131). Moreover, the use of less complex matrices allows for efficient flow rates since the flow rate is mostly dictated by the nature of deposited food matrix components on the surface of the membrane (172). As a result simpler matrices (tap water and clear apple juice) were used for filtration experiments following enrichment. Enrichment of water and juice matrices spiked with a maximum of 15 CFU of the target in 250 ml broth for 4 hrs allowed for the capture of at least 100 CFU of the target released from the membrane using IMS (Fig 9). Similar capture efficiencies were observed when the target was enriched in the presence of either *Salmonella* Typhimurium or non-pathogenic *E. coli* that were spiked to the same amount (Fig 10-12). Capture experiments also showed that the number of cells captured was dependent on the target isolate with serotypes O26 and O111 having lower recovery with higher initial inoculum concentrations (Fig 9, 10, and 12), suggesting that these serotypes may have slow growth patterns under the enrichment conditions tested. These results demonstrated that an enrichment period of 4 hrs is sufficient for the detection of the target STEC via the combination of membrane filtration and IMS with the mAb utilized in these studies. Additionally these antibodies were implemented in the direct visualization of the target STEC following a short enrichment (4 hrs), filtration, and capture with IMS. All serotypes being tested were easily visualized following elution and probing with the mAb against the target STEC (Fig 15). This allows for the quick visual screening of samples of interest thereby streamlining the diagnostic process. The use of IMS prior to visual detection helps clarify the sample being tested from a majority of food matrix components and background microflora which is often considered a major interference in fluorescence based detection of food pathogens (171). Though filtration followed by direct visualization has been reported previously (166-171), the filtration of the entire culture volume after a short period of enrichment and visualization of targets

thus isolated have not been reported before. The utilization of the entire enrichment volume for the detection of STEC provides a unique advantage since most food samples are usually contaminated with very low levels of the target (173), and the dilution of the sample for enrichment further dilutes the STEC contaminant. Additionally, the short enrichment period also allows for resuscitation and growth of these STEC thereby enabling the cells to reach or exceed detection limits of assays like IMS, microscopy, and even PCR (174-176). It is also interesting to note that a study that coupled secondary detection following IMS, was able to lower the detection limit from 10^4 CFU/ g of meat to 10 CFU/ g following a 6 hr enrichment period (177). Though our study dealt with simpler matrices, it was demonstrated that a shorter enrichment time coupled with filtration and IMS followed by direct plating or immunofluorescence microscopy was able to obtain detection limits of 0.6 CFU/ ml of sample (i.e., maximum of 15 CFU in 25 ml of sample). However, the method presented here is mainly limited by the filterability of the matrix being tested, and as a result it cannot be applied to all samples in a food testing environment. Furthermore, all the matrices tested were inoculated with bacteria from pure culture and therefore do not represent the true state of target cells (injured, stressed) usually found in food matrices (178). Therefore, a longer enrichment time may be necessary to obtain similar results under the conditions tested. It should be noted however, that 4 hrs for enrichment time was chosen due to the fact that when 6 hr enrichments were performed it was difficult to enumerate the number of cells captured with IMS ($>10^4$ CFU – data not shown). Given the fact that at least 100 CFU was recovered from an enrichment culture of samples spiked with ≤ 15 CFU, it is likely that a 4 hr enrichment may be sufficient to increase the cell counts to detectable levels with IMS for bacteria subjected to injury and stress in real world samples. However to reduce the chance of obtaining false negative results,

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the extension of the enrichment time to 6 hrs would be recommended to accommodate the resuscitation of slow growing serotypes like O26 and O111.

3.1 INTRODUCTION

3.1.1 FLOW THROUGH IMMUNOASSAY

The flow through immunoassay (FIA) is an automatable offshoot of IMS that involves the immobilization of antibodies on a solid matrix such as beads or membranes. In FIA, the antigens of interest are either allowed to flow through an antibody coated membrane or a column packed with antibody coated beads. The captured target can then be detected either by plating or chromogenic means (179, 180, 181). IMS has been used with success when testing for foodborne pathogens (132, 171), however samples have to undergo long enrichment steps to meet the requirement for high bacterial densities and low testing volumes. Various groups have tried to develop different solid matrix and membrane based methods; however, clogging when complex matrix suspensions are processed, and inhibition of capture by matrix components limits their use (182, 131). The use of larger bead sizes have been theorized to help circumvent this issue and their use has shown potential in resolving issues like clogging with varying degrees of capture efficiencies (182). One study reported that polystyrene beads (125 μm dia.) coated with passively adsorbed antibody fragments were able to capture up to 75% of their target (*Pseudomonas aeruginosa*) from a fat free milk sample (131), while another group reported that large glass beads (>3mm) coated with antibodies were able to capture 13% of target spores in environmental water samples (183). The ability of larger (150-212 μm) antibody coated beads to bind O157 STEC was qualitatively demonstrated by another group using a microfluidic chamber (184). These studies have provided sufficient evidence to suggest that using larger bead sizes could provide an effective means to capture target STEC from enriched food samples.

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3.1.2 EXPERIMENTAL DESIGN

In the study described in this chapter, two different solid matrices were tested to determine which would serve as a potential candidate for a flow through assay designed to capture STEC using the monoclonal antibodies described earlier. The two solid matrices that were tested were glass beads (~300 µm in diameter) and a range of sizes of polystyrene beads (100 – 3175 µm in diameter). In this study, the surface of both matrices were coated with protein G, following which monoclonal antibodies against O157 were coupled to the beads. Approximately 1000 CFU of an O157 isolate was then added to the beads and the capture efficiency of the beads was determined by plating the eluted target cells and the beads on LB agar. In the case of polystyrene beads the first set of experiments were performed by passively adsorbing protein G on to the surface of polystyrene beads of varying sizes. Once an optimal size of beads was determined, the ability of the passively adsorbed protein G coated polystyrene beads to capture the target was tested with four samples: (1) 1 ml of a bacterial suspension in PBS containing 10^3 CFU of the target, (2) 250 ml of a bacterial suspension in PBS containing 10^3 CFU of the target, (3) 250 ml of an enrichment culture that contained 225 ml of mTSB and 25 ml of spiked tap water (~10 CFU of O157) following 4 hrs of enrichment, and (4) 250 ml of a 1:1 dilution of mTSB in PBS spiked to a final target concentration of 10^3 CFU. Additionally, a series of experiments were performed to determine if covalently conjugating protein G to the polystyrene beads could improve capture efficiency under the conditions described above.

3.2 MATERIALS AND METHODS

3.2.1 BACTERIAL CULTURES

Bacterial cultures used for capture experiments were obtained by inoculating a single colony of O157:H7 in 5 ml of LB medium and incubating the cultures overnight at 37°C with agitation (250

rpm). *E. coli* BL21(DE3) harboring an expression vector (pPG) coding for the protein G IgG binding domain fused N terminally to enhanced green fluorescent protein (eGFP), and a 6×His-tag (M. Lin and H. Dan, unpublished data) were grown in LB medium supplemented with 50 µg/ml of kanamycin and incubated at 37°C with agitation (250 rpm). All enrichment of water samples were performed in mTSB at 37°C with agitation (250 rpm).

3.2.2 CHEMICAL REAGENTS AND MONOCLONAL ANTIBODIES

The surface of glass beads was activated using a 2% (v/v) (3-aminopropyl) triethoxysilane (APTES, Sigma Aldrich) in acetone. Amino succinic acid-PEG-amido succinic acid (ASA-PEG-ASA, Laysan Bio. Inc.) was used as a spacer at a final concentration of 10 mM in a 0.1 M solution of 2-(*N*-morpholino) ethanesulfonic acid (MES, Sigma Aldrich) at pH 4.5. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Fluka) along with *N*-Hydroxysuccinimide (NHS, Thermoscientific) was then used as a cross linker at a final concentration of 5 mg/ml (each) in 0.1 M MES. A 50 mM buffer solution of MES at pH 6.1 was used to passively adsorb recombinant protein G onto the surface of the polystyrene beads. A 0.2 M solution of 1-fluoro-2-nitro-4-azidobenzene (FNAB) in methanol was used to photoactivate the surface of polystyrene beads. A 100 mM carbonate/bicarbonate buffer was used to bind recombinant protein G to the activated polystyrene beads. All dilutions of bacterial cultures, washes and resuspension of beads during capture were performed in PBS (pH 7.4). PBS containing Triton X (0.2% v/v) and Tween 20 (0.05% v/v) (PBSTT) served as a wash buffer, and a diluent for the blocking buffer and for the dilutions of monoclonal antibodies used for western blotting. PBS containing Tween 20 (0.1% v/v) (PBST) served as a wash buffer, and a diluent for the blocking buffer and for the dilutions of monoclonal antibodies used for ELISA. All capture experiments were performed using a 1 in 40 dilution in PBS (pH 7.4) of a monoclonal antibody against the O antigen of serotype O157

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designated M3475. Commercially acquired IgG elution buffer (Thermoscientific) consisting of an amine solution at pH 2.8 was used to elute captured bacteria.

3.2.3 BEADS AND COLUMNS

Protein G coated Dynabeads (Thermofisher) were used for capture studies. M-270 Epoxy Dynabeads (Thermofisher) were also coated with recombinant protein G for capture studies. Four different diameter sizes of polystyrene beads were tested: 105-125 μm , 200-300 μm , 355-425 μm , and 3175 μm (Polysciences Inc.). Acid washed 300 μm (diameter) glass beads were obtained from Sigma. A 2 ml spin column fitted with a 30 μm frit, along with a 50 ml column adapter were used for flow through assays (Pierce).

3.2.4 EXPRESSION AND PURIFICATION OF PROTEIN G

A single colony of *E. coli* BL21(DE3) containing pPG was picked from a kanamycin (30 $\mu\text{g}/\text{ml}$) supplemented LB agar plate and used to inoculate 50 ml of kanamycin (50 $\mu\text{g}/\text{ml}$) supplemented LB broth and grown overnight at 37°C with agitation (250 rpm). The following day a 1 in 100 dilution of the overnight culture is prepared in 2 L of fresh kanamycin supplemented LB broth. The suspension was then incubated at 37°C with agitation (250 rpm) until the culture reached an optical density (OD_{600}) between 0.4 and 0.6. The expression of the recombinant protein G was then induced over three hours with the addition of IPTG (Isopropyl-beta-D-thiogalactopyranoside, Gold Bio.com) to a final concentration of 1 mM. The bacterial culture was then stored overnight at 4°C and the cells were pelleted at 8000 rpm for 20 min. The cell pellet was resuspended in 20 ml of PBS and frozen at -20°C until further processing. The pellet suspension was then thawed at room temperature and another 30 ml of PBS containing PMSF (phenylmethylsulfonyl fluoride, Sigma Aldrich) and an ethylenediaminetetraacetic acid (EDTA) free protease inhibitor tablet

(Thermoscientific) was added to the pellet suspension to obtain a final concentration of 1 mM of PMSF. The cells were kept on ice for 30 min and then lysed using a French press (1500 psi). The lysate was then centrifuged and imidazole was added to the supernatant to a final concentration of 2 mM. The lysate was then added to Ni-NTA resin (Qiagen) and allowed to bind overnight at 4°C with end over end mixing. The following day the resin was packed into a column, washed with 25 ml of wash buffer (50 mM NaH₂PO₄.H₂O, 300 mM NaCl, 20 mM imidazole, pH 8.0) and protein G eluted in 3 ml fractions with elution buffer (50 mM NaH₂PO₄.H₂O, 250 mM imidazole, pH 8.0). Elution fractions were then pooled, concentrated, and buffer exchanged against PBS using an Amicon protein concentrator (MWCO 50 kDa). The protein concentration was then quantified using a Bradford assay with BSA as a standard (Bio-Rad protein assay kit).

3.2.5 WESTERN BLOTTING

Concentrated protein G sample (6 µg per lane) was run on two separate lanes of an SDS PAGE (4 % stacking and 12% resolving gel) along with the protein standard (Precision Plus, Bio-Rad) (180 V, 40 min). One part of the gel consisting of a set of the sample and the protein standard were stained using Coomassie blue (Bio-Rad) for 2 hrs and then destained overnight with de-staining buffer (40% (v/v) methanol, 10% (v/v) acetic acid in water). The other set was then transferred onto a nitrocellulose membrane via a semi dry transfer apparatus (Trans-Blot, Bio-Rad) (15 V, 30 min), and blocked with 3% IgG free BSA (w/v) in PBSTT for 1 hr at room temperature. The membrane was then probed with a 1 in 2000 dilution of a peroxidase conjugated goat anti mouse antibody in PBSTT for 1 hr at room temperature. The membrane was then washed 3 times with PBSTT for 5 minutes and developed using the HRP conjugate substrate kit (Bio-Rad) for 10 min. The membrane was then washed in water for 10 min, and allowed to air dry.

3.2.6 FUNCTIONALITY OF RECOMBINANT PROTEIN G IN CAPTURE

Recombinant protein G was conjugated on the surface M-270 epoxy Dynabeads according to manufacturer's instructions (Thermoscientific). Briefly 2 mg of recombinant protein G was added to $\sim 2 \times 10^9$ beads in 900 μ l of PBS (pH 7.4) containing ammonium sulfate at 1 M final concentration. The beads were then incubated for 48 hrs at 4°C with end over end mixing and then washed 5 times (5 min) with PBS containing 0.1% (w/v) of IgG free BSA. After washing, a 1 in 40 dilution of the monoclonal antibody (TCF) was added to the beads and incubated at room temperature for 30 min with end over end mixing. The beads were then washed 3 times with 1 ml of PBS. To test the functionality of the recombinant protein G in binding the monoclonal antibody, a 1 ml of bacterial suspension in PBS ($\sim 10^3$ CFU) was added to the beads and incubated at room temperature for 30 min. Following 3 washes with 1 ml of PBS, captured bacteria were eluted 3 times using 200 μ l of IgG elution buffer (Thermoscientific) and neutralized with 20 μ l of 1 M Tris (pH 9.0). The elution fractions were then plated on LB agar plates and incubated overnight at 37°C. The capture of these beads were compared to the capture of commercial protein G coated beads.

3.2.7 ACTIVATION AND COVALENT CONJUGATION OF PROTEIN G ON TO GLASS BEADS

In order to activate the glass bead surface, 50 g of the glass beads (300 μ m) were weighed out into a glass beaker; 50 ml of the APTES solution was added to the beads and allowed to incubate at room temperature for 1 min in a fume hood. The solution was then decanted and 50 ml of fresh APTES solution was added to the beads and allowed to incubate for 30 min at room temperature. Following incubation the beads were rinsed 5 times with 50 ml of acetone, and then washed twice with 50 ml of methanol. The beads were then cured at 80°C for 30 min to ensure crosslinking, following which the beads were air dried and aseptically stored at room temperature. The beads (20 g) were then pegylated with 20 ml of the spacer ASA-PEG-ASA solution. The beads were

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incubated at room temperature for 2 hrs with gentle agitation (150 rpm). The solution was then decanted and the beads were washed with a 100 ml of PBS (pH 7.4) 5 times, followed by 2 washes with 100 ml of Milli Q deionized water. The beads were then treated with 20 ml of the cross-linker solution (EDC and NHS 5 mg/ml each in MES) and allowed to incubate at room temperature for 30 min with gentle agitation (150 rpm). The beads were washed 5 times with 50 ml of Milli Q deionized water and stored at 4°C in 20 ml of water. Recombinant protein G was then conjugated to the activated beads by adding 4 mg of the protein G in 3 ml of PBS to 2 g of the activated beads. The beads were then incubated overnight at 4°C with end over end mixing, and then further incubated at room temperature for 2 hrs with end over end mixing. The reaction was stopped with the addition of 150 µl of 1 M Tris (pH 7.5) followed by incubation at room temperature with end over end mixing for 30 min. The solution was then decanted, and the beads were washed 5 times with 3 ml of PBS. The beads were blocked with 3 ml of 3% (w/v) of IgG free BSA in PBS for 30 min at room temperature with end over end mixing. The beads were then washed 5 times with PBS and were stored in 3 ml of sterile PBS at 4°C. BSA coated beads were prepared in a manner similar to those coated with protein G.

3.2.8 PASSIVE ADSORPTION OF PROTEIN G ONTO POLYSTYRENE BEADS

Polystyrene beads (100 µm, 200 µm, 300µm, and 3175 µm in diameter) were used for capture experiments. The 100 µm, 200 µm, and 300µm beads were coated with purified recombinant protein G by adding 0.5 mg of protein G in 1 ml of 50 mM MES buffer (pH 6.1) to 0.05 g of the beads. Five of the largest polystyrene beads (3175 µm) were treated with 1 ml of the recombinant protein G (1 mg/ml). The beads were allowed to incubate overnight at 4°C with end over end mixing followed by incubation at room temperature with end over end mixing for 1 hr. The supernatant was then removed and 1 ml of blocking buffer consisting of 1 mg/ml IgG free BSA in

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PBS was added to the beads and incubated for 1 hr at room temperature. BSA coated beads were prepared in a manner similar to that described for coating the beads with protein G.

3.2.9 PHOTOACTIVATION AND COVALENT CONJUGATION OF PROTEIN G ON TO POLYSTYRENE BEADS

Polystyrene beads (100 μm in diameter) were photoactivated by adding 100 μl of a 0.2 M solution of FNAB in methanol to 0.05 g of polystyrene beads. The bead suspension was then transferred into the wells of a 12-well plate. The methanol was allowed to evaporate for 15 min, in the dark in a fume hood. The beads were then exposed to a UV source (365 nm) for 20 min, and were then washed multiple times with methanol to remove any unbound FNAB. The beads were transferred to a 50 ml tube and the methanol was allowed to evaporate in the fume hood. The recombinant protein G was then conjugated to the activated beads by adding 0.5 mg of freshly prepared protein G in 1 ml of carbonate-bicarbonate buffer (pH 9.6) to the beads. The beads were then incubated at 37°C with light agitation (150 rpm) for 1 hr. The supernatant was removed and the beads were blocked with 3% IgG free BSA (w/v) in PBS for 1 hr at 37°C with gentle agitation (150 rpm). The supernatant was removed and the beads were washed 5 times with 1 ml PBS containing 0.1% Tween 20. BSA coated beads were prepared in a manner similar to that described for coating the beads with protein G.

3.2.10 IMMUNOASSAY BASED CONFIRMATION OF PROTEIN G COATING

An immunoassay was performed on the protein G coated beads to confirm the presence of protein G on the bead surface. The amount of beads tested was the same amount that was to be used in capture experiments. Briefly, 0.15 g of the glass beads, 0.05 g of the polystyrene beads (100 μm , 200 μm , and 300 μm in dia.), 5 beads (3175 μm in dia.), and 50 μl of commercial protein G Dynabeads were tested. All protein G and BSA coated beads were blocked with 3% IgG free BSA

(w/v) in PBS. The FNAB treated beads were incubated for 1 hr at 37°C, while all other beads were incubated for 1 hr at room temperature. The supernatant was removed and a 1 in 2000 dilution of a peroxidase conjugated goat anti-mouse antibody was added to the beads and incubated for 1 hr as described for the blocking step. The beads were then washed 5 times with PBS and developed with a substrate solution of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (0.5 mg/ ml of ABST in 0.05 M citrate buffer (pH 4.5), 0.03% (v/v) peroxide) for 10 min. The supernatant was then transferred to the wells of a 96-well plate and diluted when required in citrate buffer (0.05M, pH 4.5) and read using a spectrophotometer at 415 nm. The results were compared to the immunoassay results obtained for 50 µl of protein G coated Dynabeads that served as the positive control.

3.2.11 CAPTURE AND RECOVERY OF O157 USING GLASS BEADS

Blocked protein G coated glass beads were weighed out into a 1.5 ml microcentrifuge tube, and a 1 in 40 dilution of the monoclonal antibody (TCF) against O157 in PBS (pH 7.4) was added to the beads, and incubated at room temperature for 30 min with end over end mixing. The beads were then washed 3 times with PBS. Overnight cultures of the isolate of serotype O157 being tested were diluted to an optical density of 1 ($1 \text{ OD}_{600} = 1 \times 10^9 \text{ CFU/ ml}$) and then further diluted in PBS to a concentration of 10^3 CFU/ ml . The cell concentration was confirmed by plating 100 µl of this suspension on duplicate LB agar. One ml of bacterial suspension in PBS containing 10^3 CFU was then added to the beads and was allowed to incubate for 30 min with end over end mixing at room temperature. The supernatant was removed and the beads were washed 3 times with PBS. Bound bacteria were eluted 3 times each with 200 µl of IgG elution buffer (Thermoscientific), and the fractions were neutralized with 20 µl of 1 M Tris (pH 9.0). The neutralized fractions were plated on LB agar plates. The beads that had been submerged in LB broth and the LB agar plates were

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incubated overnight at 37°C. The capture results obtained using glass beads were compared to results obtained with commercial protein G coated Dynabeads used under the same conditions described above.

3.2.12 CAPTURE USING PASSIVELY ADSORBED PROTEIN G POLYSTYRENE BEADS

Overnight cultures of the isolate of serotype O157 being tested were diluted to an optical density of 1 ($1 \text{ OD}_{600} = 1 \times 10^9 \text{ CFU/ml}$) and then further diluted in PBS to a concentration of 10^3 CFU/ml . The cell concentration was confirmed by plating 100 μl of this suspension on duplicate LB agar. Protein G coated polystyrene beads (0.05 g for 100- 300 μm beads and 5 beads for 3175 μm beads) blocked with 3% (w/v) IgG free BSA in PBS were coated with monoclonal antibodies by adding a 1 in 40 dilution of the monoclonal antibody(TCF) against O157 in PBS to the beads. The beads were then incubated at room temperature for 30 min with end over end mixing. The beads were then washed 3 times with PBS and 1 ml of a bacterial suspension containing 10^3 CFU of the target cells was added to the beads. The beads were then incubated at room temperature for 30 min with end over end mixing. Following incubation, the beads (100- 300 μm beads) were washed 3 times with 1 ml of PBS and the captured bacteria were eluted in three fractions each using 200 μl of IgG elution buffer (Thermoscientific), and neutralized with 20 μl of 1 M Tris buffer (pH 9.0). The neutralized fractions were plated on LB agar plates and the 100 μm and 200 μm beads were also plated on LB agar plates. The 3175 μm polystyrene beads were eluted with 600 μl of IgG elution buffer (Thermoscientific) and neutralized with 60 μl of 1M Tris (pH 9.0). The neutralized fractions were plated on LB agar plates. The 300 μm and the 3175 μm beads were submerged in LB media. All plates and beads submerged in media were incubated overnight at 37°C. Capture using polystyrene beads were compared to the capture obtained when commercial protein G coated Dynabeads were used under the same conditions described above.

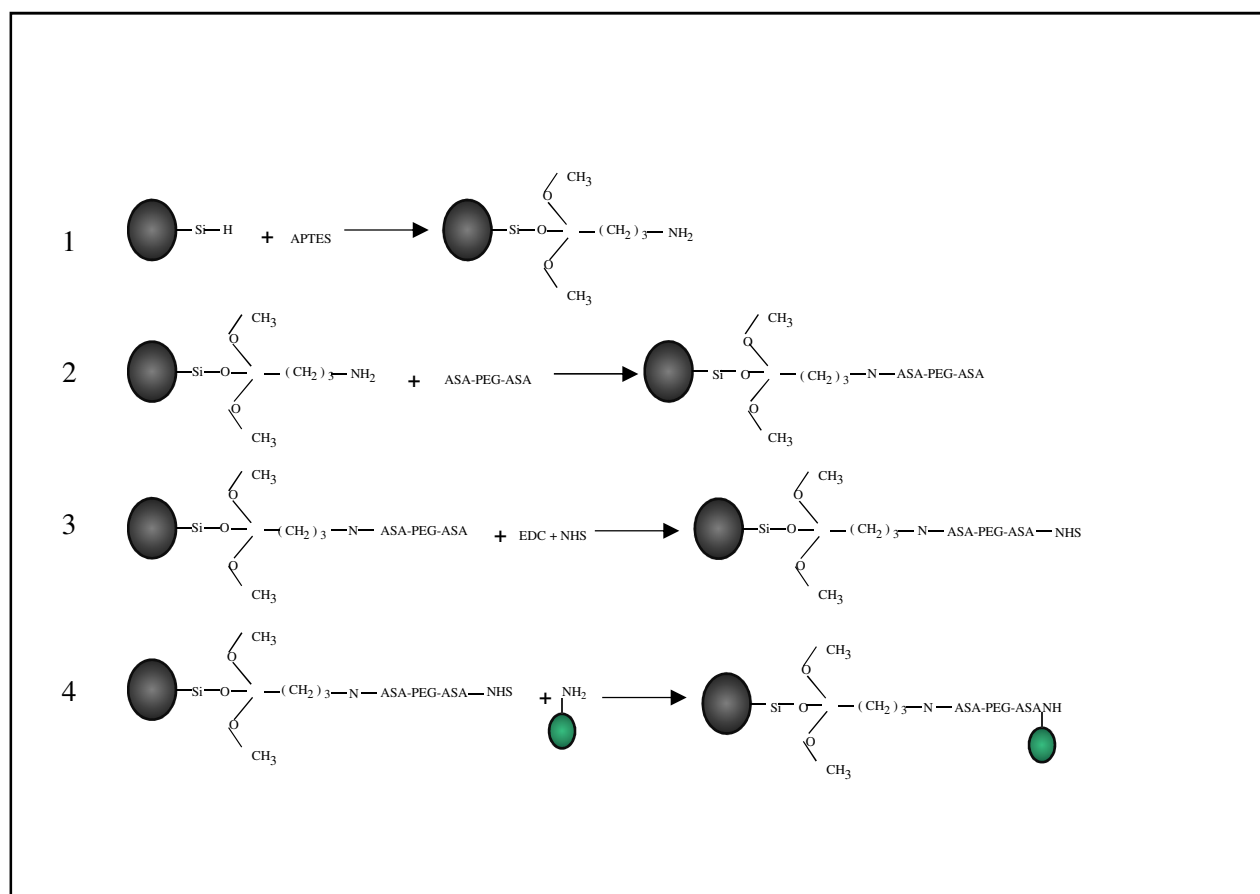


Fig 16: Activation of glass bead surface. (1) Silanization of glass is mediated by the addition of APTES. (2) ASA-PEG-ASA is added to the silanized glass and acts as a spacer. (3) EDC and NHS is added to the glass beads to create a stable amine reactive NHS ester. (4) Protein G is then conjugated to the glass via a stable amide bond.

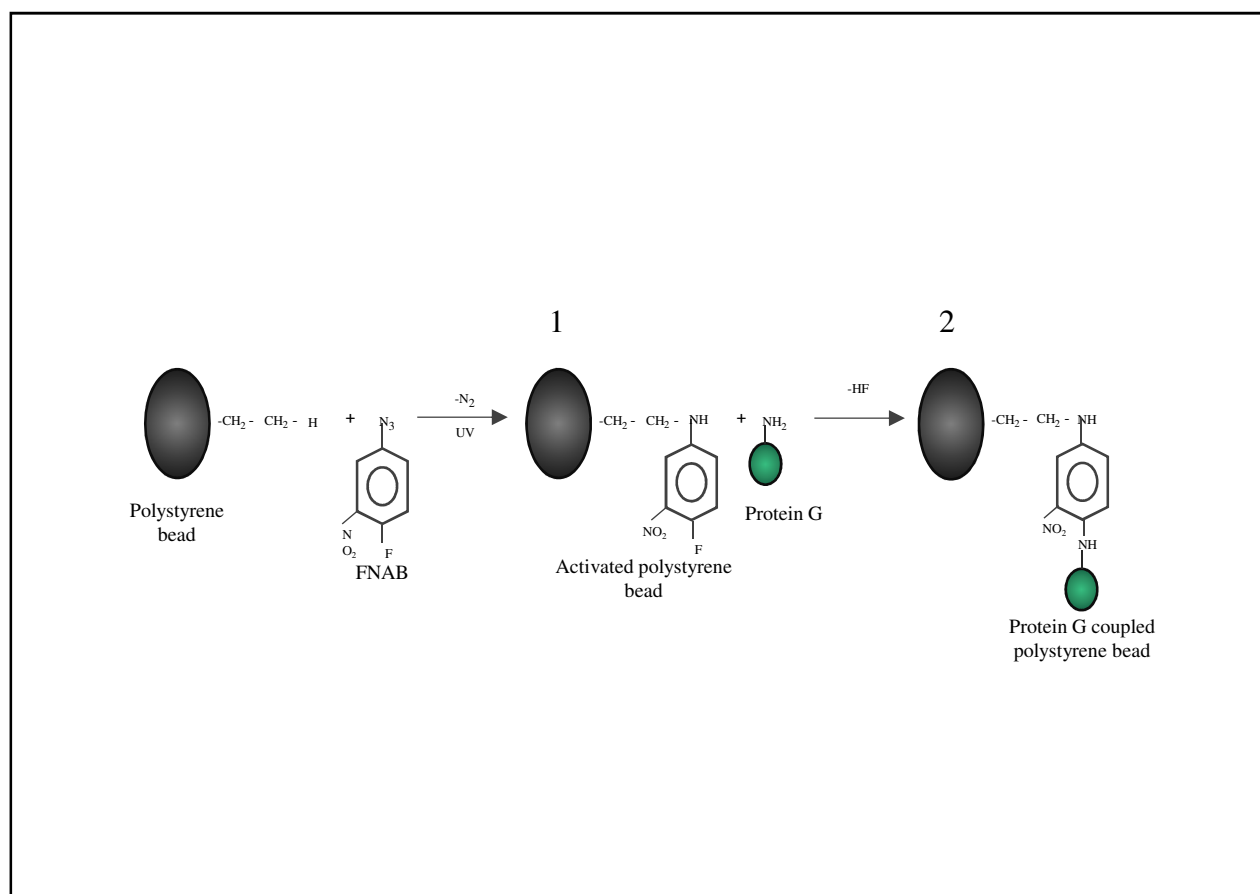


Fig 17: Photoactivation of polystyrene beads. (1) The azido group on 1-fluoro-2-nitro-4-azidobenzene (FNAB) is converted into a reactive nitrene group upon exposure to UV. The nitrene group then covalently binds to the surface of the polystyrene bead. (2) Protein G is then covalently conjugated via the reactive fluoro group on FNAB.

3.2.13 CAPTURE USING POLYSTYRENE BEADS UNDER DIFFERENT CONDITIONS

A 1 in 40 dilution of the monoclonal antibody (TCF) against the O157 serotype was added to passively coated protein G polystyrene 100 µm beads. Following the incubation and washing step (described above), the ability of these beads to capture their target was tested with four samples : (1) 1 ml of bacterial suspension in PBS containing 10^3 CFU of the target, (2) 250 ml of mTSB following a 4 hr enrichment of a water sample spiked with ~10 CFU of the target, (3) 250 ml of PBS containing 10^3 CFU of the target, and (4) 250 ml of diluted mTSB (1:1 in PBS) containing 10^3 CFU of the target. In the case of the experiment performed with a 1 ml of bacterial suspension in PBS (10^3 CFU), the sample was allowed to incubate with the beads for 30 min with end over end mixing at room temperature. The beads were then packed into a column with a 30 µm frit and 100 µl of the flow through was plated on duplicate LB agar plates. The beads were washed 3 times each with 1 ml of PBS, and 100 µl of each wash fraction was plated on duplicate LB agar plates. The captured bacteria was eluted 3 times using 200 µl of IgG elution buffer and each fraction was neutralized with 20 µl of 1M Tris (pH 9.0). The neutralized fractions were then plated on LB agar plates and the beads were resuspended in 200 µl of PBS, and the entire bead suspension was plated on LB agar plates. The plates were incubated overnight at 37°C. In the case of larger test volumes, the antibody coated beads were packed into a column with a 30 µm frit and the bacterial cells in 250 ml of media, diluted culture or PBS were allowed to flow through the column (gravity) at a flow rate of approximately 10 ml/ minute. Once the entire volume was allowed to flow through the column, the beads were processed as described above for the smaller volume experiments.

3.2.14 CAPTURE USING COVALENTLY CONJUGATED PROTEIN G POLYSTYRENE BEADS

A 1 in 40 dilution of the monoclonal antibody (TCF) against O157 in PBS containing 0.1% (v/v) tween 20 was added to 0.05g protein G coated polystyrene beads, and incubated at 37°C for 1 hr

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with gentle agitation (150 rpm). The ability of these beads to capture the target was tested with: (1) 1 ml bacterial suspension in PBS containing 10^3 CFU of the target, (2) 250 ml of mTSB following a 4 hr enrichment of a water sample spiked with ~ 10 CFU of the target, (3) 250 ml of PBS containing 10^3 CFU of the target, and (4) 250 ml of diluted mTSB (1:1 in PBS) containing 10^3 CFU of the target. In the case of the 1 ml of test sample, following the addition of the bacterial sample to the beads, the suspension was allowed to incubate for 30 min with end over end mixing at room temperature. The beads were then packed into a column with a 30 μ m frit and 100 μ l of the flow through was plated on duplicate LB agar plates. The beads were washed 3 times with 1 ml of PBS, and 100 μ l of each wash fraction was plated on duplicate LB agar plates. The beads were then resuspended in 400 μ l of PBS and the entire volume was plated on LB agar (200 μ l / plate). All plates resulting from these experiments were incubated overnight at 37°C. In the case of larger test volumes, the antibody coated beads were packed into a column with a 30 μ m frit; the bacterial cells in 250 ml of media, diluted culture or PBS were allowed to flow through the column (gravity) at a flow rate of approximately 10 ml/ minute. Once the entire volume was allowed to flow through the column, the beads were processed as described above for the smaller volume experiments.

3.3 RESULTS

3.3.1 WESTERN BLOT ANALYSIS OF RECOMBINANT PROTEIN G

The analysis with SDS-PAGE and western blots indicates the presence of a strong band at ~50 kDa, which is close to the theoretical molecular weight of the recombinant protein G fused to eGFP (46.6 kDa) (Fig 18A). The western blot result demonstrated that the recombinant protein G expressed was functional as it was able to bind to the peroxidase conjugated goat anti mouse antibody in the absence of a primary antibody.

3.3.2 FUNCTIONALITY OF RECOMBINANT PROTEIN G COATED DYNABEADS IN CAPTURE

Experiments were performed to test the ability of recombinant protein G to capture the target STEC from 1 ml of a bacterial suspension in PBS containing $\sim 10^3$ CFU of the target cells. The results indicated that the recombinant protein G coated Dynabeads were able to capture 100% of the target cells in the bacterial suspension. These results were identical to the capture observed when commercial protein G coated Dynabeads were used (Fig 18B).

3.3.3 CONFIRMATION OF THE PRESENCE OF PROTEIN G ON GLASS AND POLYSTYRENE BEADS

The immunoassay results showed that all the beads tested were coated successfully with recombinant protein G. A comparison of the immunoassay results show that 0.15 g of glass beads have ~ 1.7 times less protein G than 50 μ l of commercial protein G coated Dynabeads (Fig 19A). A comparison of the amount of passively adsorbed protein G on 0.05 g of selected polystyrene beads revealed that the 100 μ m beads had the most protein G bound to its surface (~ 1.4 times less than Dynabeads) while the 3175 μ m beads had the lowest amount of bound protein G (~ 26 times less than Dynabeads) (Fig 19B). When protein G was covalently conjugated to 0.05 g of 100 μ m

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photoactivated polystyrene beads, the amount of protein G bound to the bead surface increased by ~ 0.8 times more than the passively adsorbed counterpart (Fig 19C).

3.3.4 CAPTURE OF TARGET STEC USING PROTEIN G COATED GLASS BEADS

The ability of protein G coated glass beads to capture the target STEC from 1 ml of a bacterial suspension containing 10^3 CFU was assessed. Capture results indicate that protein G coated glass beads were incapable of capturing the target bacteria. On average the glass beads captured less than 10 CFU of the 10^3 CFU added to the system (Fig 20). Based on the poor capture results it was decided to forgo using 300 μm glass beads in further experiments

3.3.5 CAPTURE OF TARGET STEC BY A RANGE OF POLYSTYRENE BEADS SIZES

The ability of passively adsorbed protein G polystyrene beads of various sizes to capture the target STEC from 1 ml of a bacterial suspension containing 10^3 CFU was assessed (Fig 21). Capture experiments performed with the large polystyrene beads (3175 μm) indicated that these beads were not capable of capturing the target STEC. The 100 μm beads were able to capture ~ 110 CFU consistently when approximately 10^3 CFU of the target was added into the system. The 300 μm beads were able to capture ~100 CFU on average; however, the number of bacteria captured varied from experiment to experiment. The 200 μm beads were only able to capture approximately 47 CFU on average. Due to the consistency observed in the capture of the target STEC by the 100 μm beads, it was decided to use this size for all other capture studies described here.

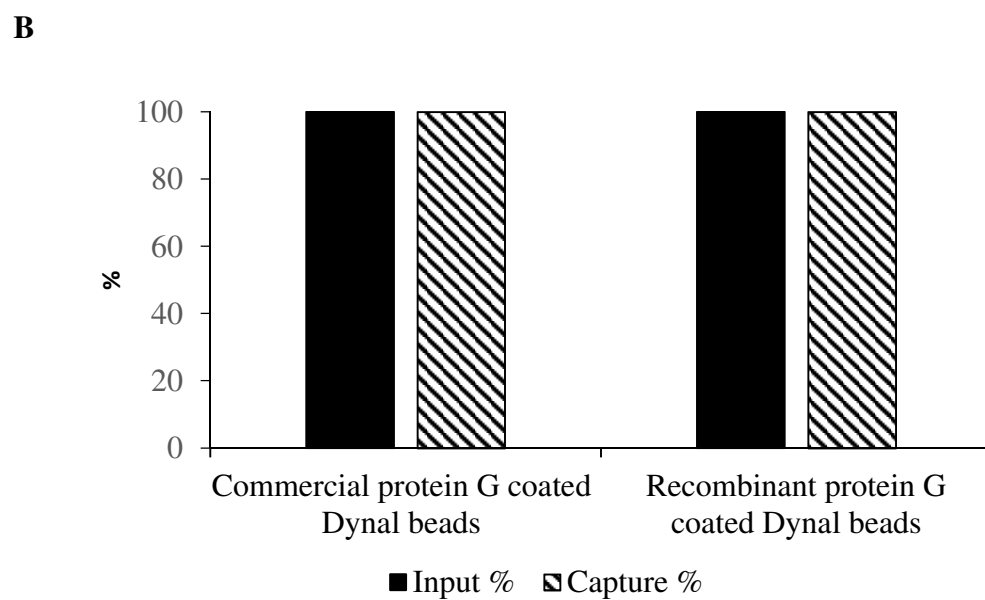
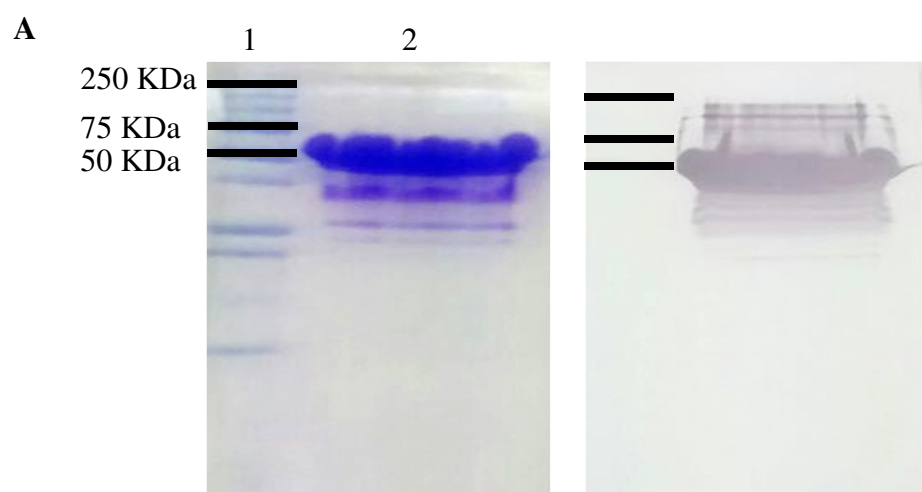


Fig 18: Protein G functionality. (A) Left: Coomassie stained SDS PAGE gel (4% stacking, 12% resolving). Well 1 contains 5 μ l of Precision plus marker (BioRad), well 2 contains 6 μ g of purified protein G fused to eGFP. The gel was run at 180 V for 40 min. Right: western blot of the purified protein G sample (6 μ g) transferred onto nitrocellulose membrane, probed with peroxidase conjugated goat anti- mouse. The purified eGFP fused protein G has a molecular weight of 46.6 KDa. (B) Percentage of target STEC (O157) captured when using commercial protein G coated Dynabeads compared to Dynabeads conjugated with recombinant protein G. The input O157 counts were normalized to 100% and the capture % were calculated based on the normalization.

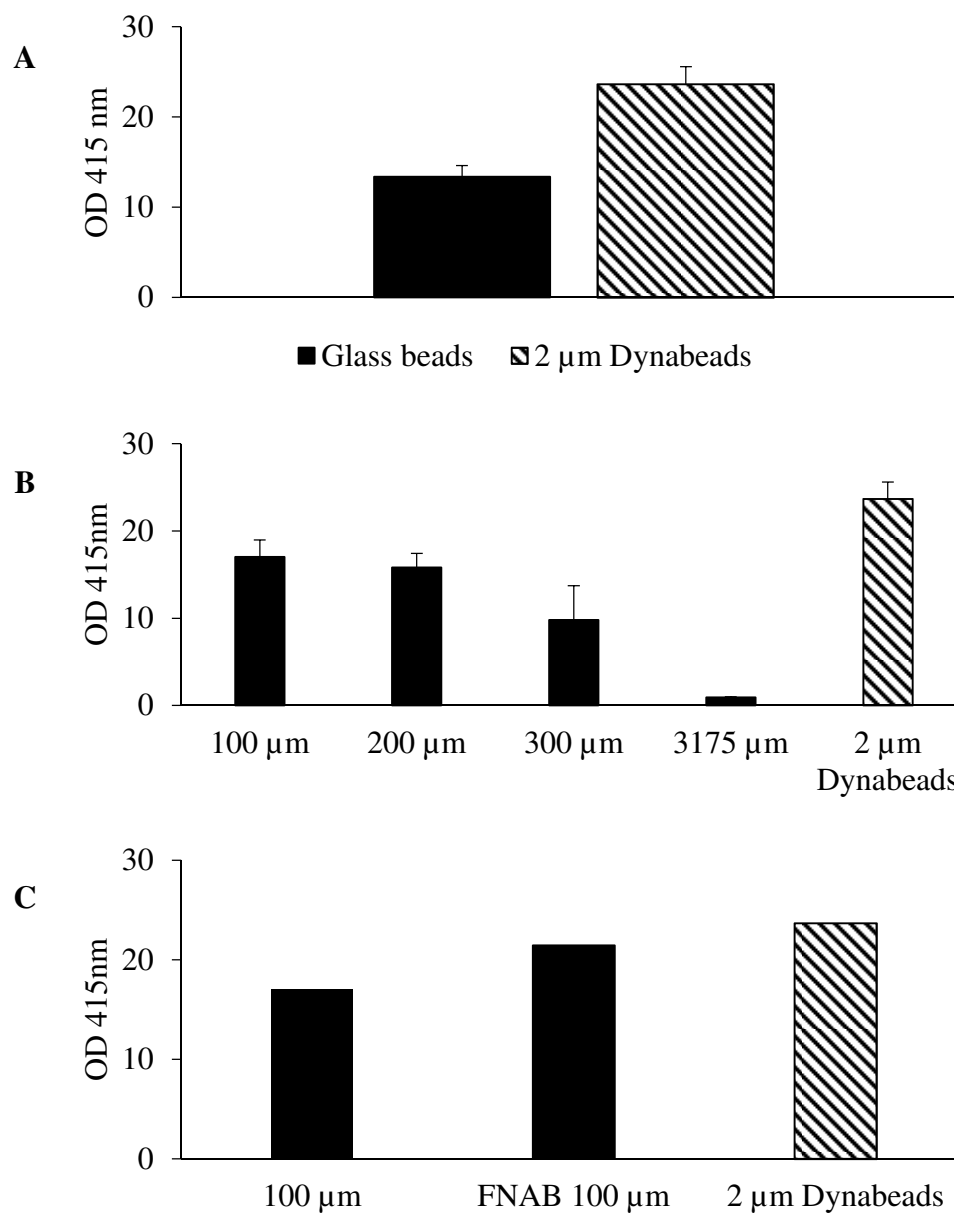


Fig 19: Immunoassay based confirmation of the presence of recombinant protein G. The protein G content on different beads compared to the protein G content found on commercial protein G Dynabeads. All beads were probed with peroxidase conjugated goat anti mouse antibody and developed using ABST (0.5 mg/ml) as a substrate. The resulting supernatant was read at 415 nm. (A) the conjugated protein G content on 0.15 g of glass beads (300 μ m in dia.), (B) the passively adsorbed protein G content on 0.05 g of 100 μ m (in dia.), 200 μ m (in dia.), and 300 μ m (in dia.) polystyrene beads, and (C) the protein G content on 0.05 g of 100 μ m (in dia.) beads that have been passively adsorbed or covalently conjugated (FNAB treated).

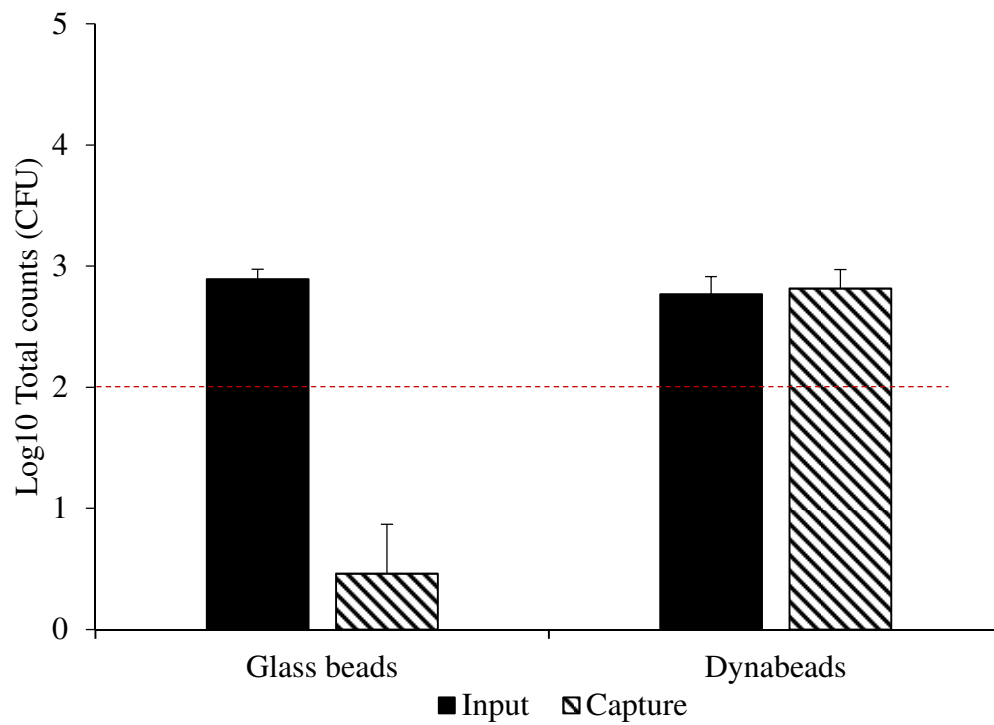


Fig 20: Capture of an isolate of serotype O157 using glass beads. Capture is represented as total counts of bacteria recovered from the capture of O157 from 1 ml of bacterial suspension in PBS containing 10^3 CFU of the target on LB agar plates. The counts obtained with glass beads are compared to the capture rates observed when commercial protein G Dynabeads are used under the same experimental parameters. Both types of beads were coated with monoclonal antibodies specific to the O157 serotype (M3475 – 1 in 40 dilution).

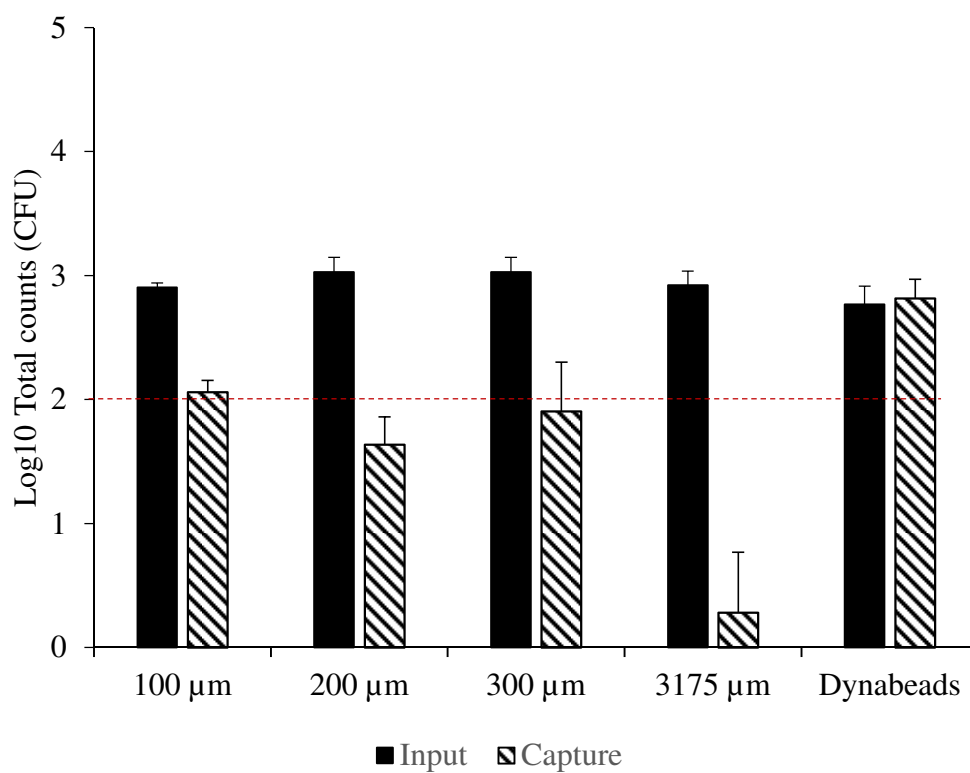


Fig 21: Capture of an isolate of O157 using different sizes of polystyrene beads. Capture is represented as total counts of bacteria recovered from the capture using different sizes of polystyrene beads from 1 ml of bacterial suspension in PBS containing 10^3 CFU of the target on LB agar plates. The counts obtained with the polystyrene beads are compared to the capture rates observed when commercial protein G Dynabeads are used under the same experimental parameters. All beads were coated with monoclonal antibodies specific to the O157 serotype (M3475 – 1 in 40 dilution).

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3.3.6 BACTERIAL CAPTURE FROM PBS USING COVALENTLY CONJUGATED AND PASSIVELY ADSORBED PROTEIN G POLYSTYRENE BEADS

The ability of passively adsorbed and covalently conjugated polystyrene beads to capture the target STEC (O157) was assessed with 1 ml and 250 ml of PBS containing 10^3 CFU of the target (Fig. 22). FNAB treated beads (100 μ m) were able to capture ~ 250 CFU of the target STEC when 1 ml of bacterial suspension containing 10^3 CFU was added into the system, while passively adsorbed protein G beads were only able to capture ~ 100 CFU. Both sets of beads were able to capture approximately ~ 100 CFU from 250 ml of PBS spiked with 10^3 CFU of the target STEC.

3.3.7 BACTERIAL CAPTURE FROM ENRICHMENT MEDIA USING COVALENTLY CONJUGATED AND PASSIVELY ADSORBED PROTEIN G POLYSTYRENE BEADS

The ability of passively adsorbed and covalently conjugated polystyrene beads to capture the target STEC serotype O157 from 250 ml of mTSB spiked with 10 CFU following a 4 hr culture enrichment was assessed (Fig 23). Additionally the ability of the two sets of beads to capture the target STEC from mTSB diluted 1:1 in PBS (v/v) and spiked with 10^3 CFU was also assessed (Fig 24). Passively adsorbed protein G polystyrene beads were not able to capture the target STEC from 250 ml of enrichment medium, and diluting the media with PBS only improved capture marginally to recover ~ 11 CFU from the expected 10^3 CFU in media following enrichment of a spiked water sample. The FNAB treated polystyrene beads were only able to capture approximately 14 CFU of the target from the expected 10^3 CFU in media following culture enrichment of a spiked water sample. However, the FNAB treated beads were able to capture ~ 92 CFU of the target cells from the diluted media spiked with 10^3 CFU of the target.

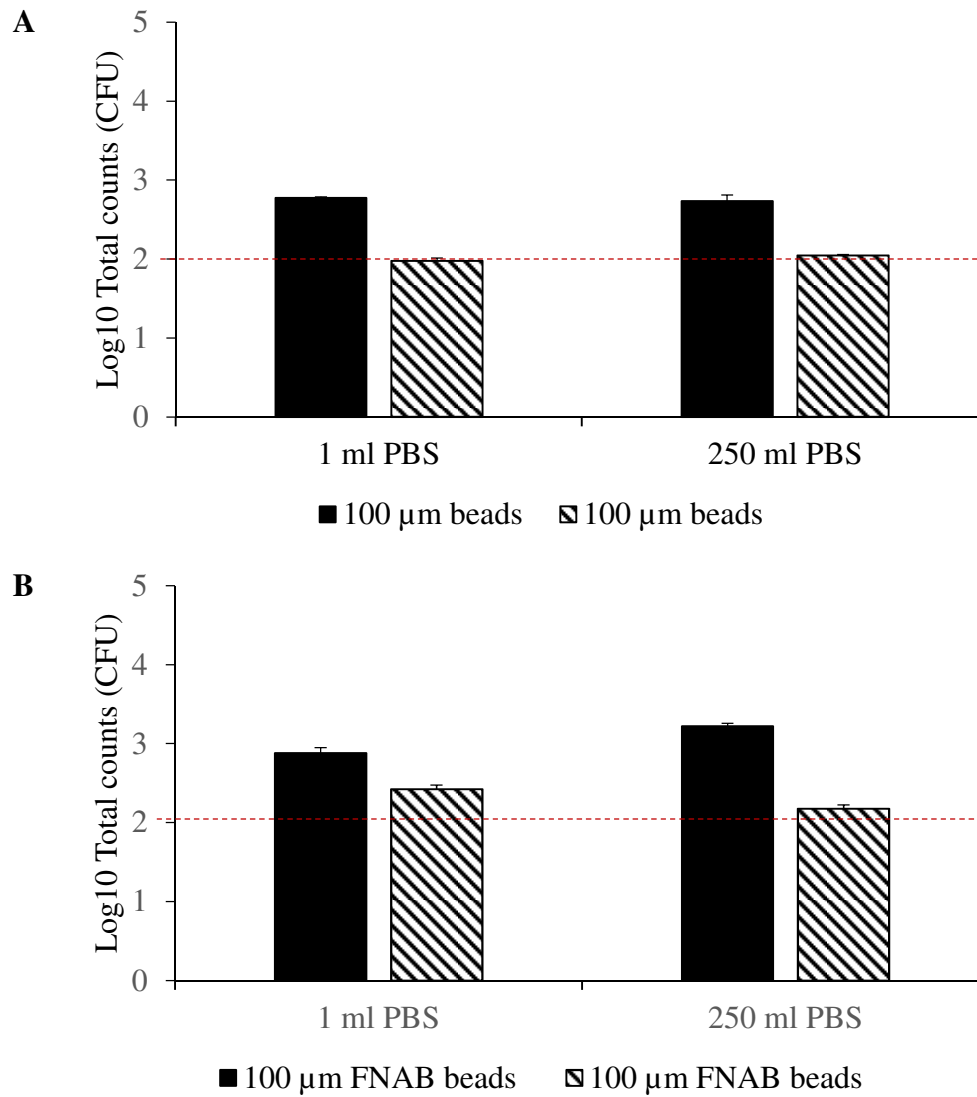


Fig 22: Comparison of capture of O157 in PBS using 100 μ m passively and covalently conjugated protein G polystyrene beads. Two different volumes of PBS were tested: 1 ml of bacterial suspension containing 10^3 CFU of the target, and 250 ml of PBS spiked with 10^3 CFU of the target. The protein G polystyrene beads were coated with monoclonal antibodies specific to the O157 serotype (M3475-1 in 40 dilution). Capture is represented as total counts of bacteria recovered from the capture experiment on LB agar plates. A- Total number of target cells recovered from both volumes tested when passively adsorbed beads are used, B- total number of target cells recovered from both volumes when FNAB treated beads (covalently conjugated) are used.

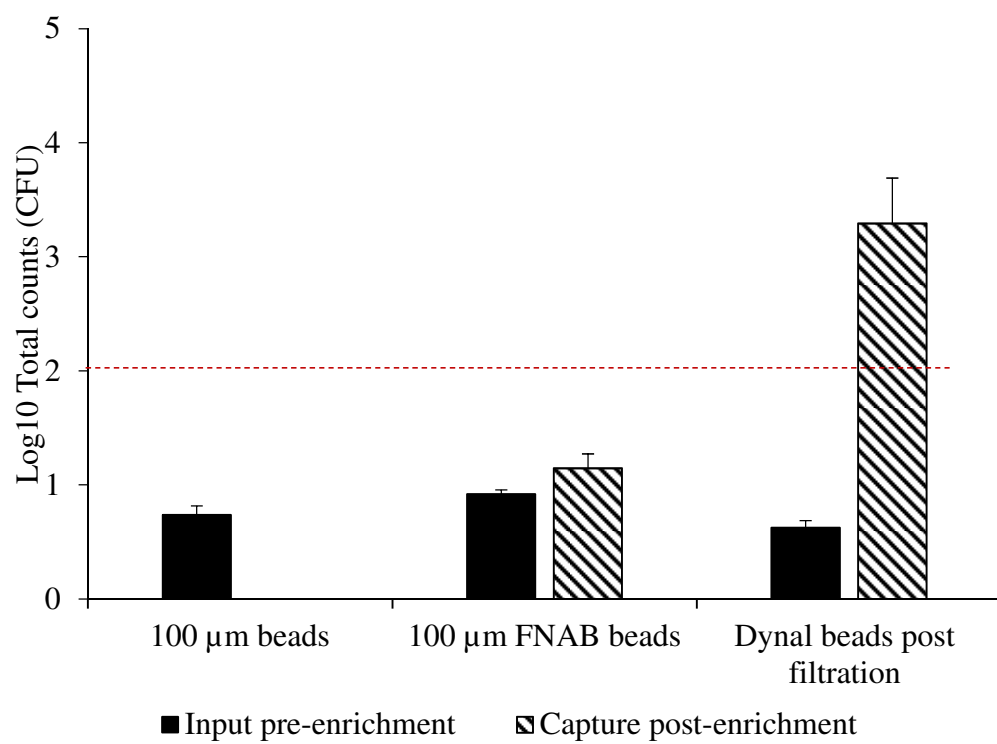


Fig 23: Comparison of capture of O157 from culture medium following a 4 hr enrichment using 100 µm passively and covalently conjugated protein G polystyrene beads. A 25 ml sample of tap water was spiked with ~ 10 CFU of the target cell and enriched in mTSB (250 ml total) for 4 hrs at 37°C. Capture was performed using passively adsorbed protein G polystyrene beads (100 µm, dia.) and covalently conjugated protein G polystyrene beads (FNAB treated 100 µm (dia.) beads). The protein G polystyrene beads were coated with monoclonal antibodies specific to the O157 serotype (M3475 – 1 in 40 dilution). Capture is represented as total counts of bacteria recovered from the capture experiment on LB agar plates. Capture rates following enrichment and filtration of spiked water samples have been included to provide approximate experimental counts captured following 4 hr enrichments of water sample when commercial protein G Dynabeads are used.

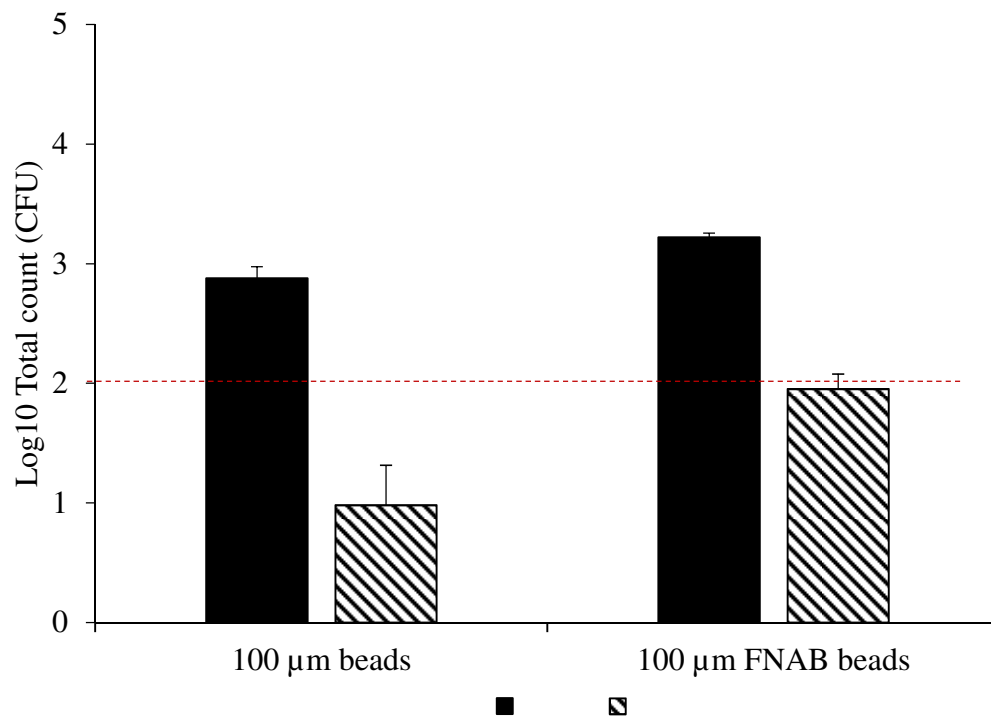


Fig 24: Comparison of capture of O157 from diluted culture medium using 100 μ m passively and covalently conjugated protein G polystyrene beads. mTSB was diluted in PBS (1:1 v/v) in a total volume of 250 ml and was spiked with 10^3 CFU of the target. Capture was performed using passively adsorbed protein G polystyrene beads (100 μ m, dia.) and covalently conjugated protein G polystyrene beads (FNAB treated 100 μ m (dia.) beads). The protein G polystyrene beads were coated with monoclonal antibodies specific to the O157 serotype (M3475 – 1 in 40 dilution). Capture is represented as total counts of bacteria recovered from the capture experiment on LB agar plates.

3.4 DISCUSSION

Flow through immunoassays have been developed to capture bacterial pathogens from diluted food matrices. However the volume from which the pathogen is detected is low, due to the fact that matrices containing high particulate matter can clog membrane and bead based assays (131). It has been suggested however that using larger beads could help circumvent issues caused by clogging (131, 183). Weimer *et al* reported that monoclonal antibody coated pegylated glass beads (3 mm) were able to capture ~ 3 times more O157 cells when compared to unpegylated beads as assessed by HRP mediated detection (185). The same group then went onto develop an automated method known as Immunoflow with pegylated glass beads that showed a capture efficiency of 1 cell of O157 from beef extract and PBS independent of the volume used. However certain intrinsic limitations existed in this study: (1) capture efficiency of these assays were based on colour development by TMB – ELISA substrate and were not confirmed with plating in the case of O157 (high OD values associated with high background microflora), (2) the exact volume of the samples used were not clearly stated (target O157), nor was it clear how the beef extract was processed and extracted, (3) the Immunoflow method was also found to be only functional within a certain range of pathogen concentrations, as a result the implementation of such a method would require the use of multiple dilutions of a matrix suspension, and (4) when plating was performed for spore samples, it was done with 1 bead per time point resulting in lower than expected counts due to the fact that sampled bead may not have bound target (183). The purpose of the work described in this chapter was to determine whether capture and recovery of target STEC cells could be achieved using beads made of either glass or polystyrene ($\geq 100 \mu\text{m}$) in a flow through system. The specificity of the beads were ensured by coating them with monoclonal antibodies raised against the LPS O antigen of serotype O157. The sizes of beads tested were chosen as they were thought

to be large enough to minimize clogging, yet small enough to allow effective sampling. The first step to this approach was to produce recombinant protein G which was used to coat the beads. This approach was pursued since protein G coated beads will enable the coating of monoclonal antibodies in an orientation that allows for optimal antigen binding. A strong band on a western blot performed in the absence of a primary antibody at ~ 50 kDa indicated that the recombinant protein G was capable of binding the antibody (Fig 18A). Additionally the ability of recombinant protein G coated beads to be used in capture experiments were assessed. The recombinant protein G coated Dynabeads were able to capture 100% of the $\sim 10^3$ CFU target O157 bacterial cells added to the system (Fig 18B). These results indicated that the recombinant protein G was functional, and was effective at capture. Glass beads (~ 300 μm) were activated in a similar manner as that described by Weimer (183), with ASA-PEG-ASA serving as a spacer. The recombinant protein G was either covalently coupled to the glass beads or was passively adsorbed onto the surface of polystyrene beads (100 μm – 3175 μm). The amount of beads tested for protein G content was the same as that used for capture (50 μl of commercial protein G Dynabeads, 0.15 g of glass beads, 0.05 g of 100 - $300\mu\text{m}$ beads, and 5 – 3175 μm beads). The presence of recombinant protein G on the surface of these beads was confirmed using an immunoassay. It should be noted that none of the beads tested had the same amount of protein G as that found on commercial protein G Dynabeads (50 μl), and this was especially true for the largest beads (3175 μm) (Fig 19). Capture experiments were then performed with 1 ml of PBS containing $\sim 10^3$ CFU of O157 cells. Protein G coated glass beads were not able to capture more than 3 CFU (on average) out of the 10^3 CFU target cells added into the system (Fig 20). Similarly the largest beads (5 beads -3175 $\mu\text{m} = \sim 3$ mm) tested were not able to capture any of the target cells (Fig 21). This was in contrast to the experiments performed by Weimer *et al* who reported a sensitivity of 1 cell of the target using

antibody coated glass beads (183). It can be speculated that these differences could be attributed by inefficient activation of glass beads resulting in insufficient protein G coating the beads. Additionally since actual counts were not performed in the study (183), it is hard to compare the results obtained here to those in the published work. The three other sizes of the passively adsorbed protein G polystyrene beads were able to capture ~ 100 CFU of the 10^3 CFU target cells put into the system (Fig 21). The results are consistent since the beads were confirmed to be coated with approximately the same amount of protein G by immunoassay. The capture results obtained for the 100 μm beads were more consistent than those obtained for 200 μm and 300 μm polystyrene beads. This could be due to the smaller size and higher surface area of the 100 μm beads, allowing for more uniform coating of recombinant protein G. As result of the consistency observed, the 100 μm beads were used for further experiments. Passively adsorbed protein G beads failed to capture the target STEC from 250 ml of enrichment culture following a 4 hr enrichment of a water sample spiked with ~ 10 CFU of O157 target cells (Fig 23). Two factors were thought to have contributed to this decrease in capture: (1) the volume of the sample being tested could be too high and could potentially shear/ wash off the protein G from the surface of the beads, and (2) components in the media interfered with capture. In order to test what was causing the drop in capture, experiments were performed using 250 ml of PBS spiked with 10^3 CFU of O157 (Fig 22A), and 250 ml of mTSB diluted in PBS (1:1, v/v) spiked with 10^3 CFU of O157 (Fig 24). Additionally protein G was covalently conjugated to polystyrene beads and tested in a similar manner as its passively adsorbed counterpart in order to ensure that the possible loss of protein G did not play a role in the reduction of capture. The passively adsorbed protein G polystyrene beads were able to capture ~ 100 CFU of the target cells from 250 ml of PBS (Fig 22A) which suggests that the loss of protein G is not likely the reason for the reduction in capture. Passively adsorbed beads were not able to

capture the target from enrichment media, however approximately ~ 11 CFU of the target were recovered from the diluted enrichment media (Fig 24). These results suggest that media components play a major role in the reduction of capture efficiencies by either interfering with the interaction between the antibody and the antigen or by disrupting the interactions between protein G and the polystyrene or possibly by a combination of both events. Interestingly, though covalently conjugated protein G polystyrene beads had very similar protein G content when compared to the commercial protein G coated Dynabeads, these beads were only able to capture ~ 250 CFU from 1 ml of PBS containing 10^3 CFU of O157. The capture efficiency was reduced to ~ 100 CFU when the test volume was increased to 250 ml of PBS (Fig 22B). These results suggest that the flow through system reduces contact between the target cells and the antibody and therefore reduces capture rates. Approximately 14 CFU of the O157 cells were recovered from 250 ml of media following a 4 hr enrichment of a water sample spiked with 10 CFU of the target cells by the covalently conjugated polystyrene beads (Fig 23); the capture rates were restored to ~ 100 CFU when the media was diluted in PBS and spiked with 10^3 CFU of O157 (Fig 24). Taken together, these results suggest that capture in this system is hindered by the interference of medium components and the contact time between the sample and the beads. Additionally the results suggest that covalently coupled protein G beads maybe more efficient at capture than one that utilizes passive adsorbed protein G beads. It is therefore plausible that the covalently conjugated protein G polystyrene beads could serve as a promising candidate for flow through capture assays. Further testing of this type of beads with other food matrices would be necessary to optimize this procedure for a range of test samples.

4.1 GENERAL DISCUSSION

Diarrhea associated HUS was first reported to be caused by STEC in 1983 (92). Since then, STEC infections have been recognized as the leading cause of pediatric acute renal failure (186). Serotype O157: H7 is one of the most well-known STEC having been involved in multiple outbreaks worldwide (93). However, over the past decade there has been a growing recognition of the role that non-O157 serotypes play in causing STEC related illnesses (13, 70, 94). An example of note was the outbreak in Germany caused by the consumption of sprouts contaminated with serotype O104:H4 that affected over 3500 people, leading to 54 deaths (187). STEC infections are often caused by the consumption of contaminated food and water (26). As a result the detection of these pathogens in food and water is paramount to preventing STEC related outbreaks.

The current methods implemented at the CFIA for the detection of clinically significant STEC (MFHPB-10 and MFLP-52, Health Canada) are usually preceded by a lengthy enrichment step (18-24 hrs). Following enrichment, small volumes of the culture are utilized for detection of the target STEC by a combination of PCR, IMS, and culture based methods. The procedures are time consuming and tedious, requiring up to 5 days before a result can be obtained. Furthermore inhibitors of PCR and high background microflora present in complex food samples can confound results obtained from these procedures (156-162).

The overall objective of this thesis was to develop a method that would streamline the detection procedure by reducing enrichment times, followed by the use of immobilized monoclonal antibodies specific to the O antigen of clinically significant STEC to capture their targets from the entire enrichment culture. Reduced enrichment times will in effect shorten the overall testing turnaround times and could also help overcome the inhibition of STEC growth as a consequence

of high background microflora concentrations (160, 161). Two approaches that were undertaken have been presented in this thesis. The first approach involved the use of a gradient filtration system to clarify enrichment culture of food matrix components, followed by the capture of the target STEC. The aim of this approach was to determine if the gradual removal of food matrix components could be achieved, thereby concentrating the target cells and minimizing its interference in successive capture (IMS) and visualization procedures. Most reports that have studied filtration of food matrix components have performed preliminary single coarse filtrations, and their results indicate that the nature of the matrix being studied dictates the volume that can be processed in such a system (166, 167). The results generated in the second chapter of this thesis suggested that the same is true even when a series of membranes are used to clarify more complex food matrices. The results indicate that cultures resulting from the enrichment of beef and produce cannot be processed in this manner. However, filtrations performed with simpler matrices like water and juice have shown that capture performed with mAb coated Dynabeads following a short enrichment step were able to isolate at least 100 CFU of the target. Furthermore, these results were found to be consistent in the presence of competitor bacterial species. Studies have shown that enrichment increases the efficiency of IMS (188), however it has also been reported that capture rates remained unchanged following either 6 hr or 24 hr enrichment for most isolates tested (189). These studies strongly suggest that an enrichment step enhances capture, however it calls to question the benefits of extended enrichment times. In the studies described in this thesis, a 4 hr enrichment period was implemented resulting in the capture and isolation of at least a 100 CFU of the target STEC. However it is important to consider that the bacterial cells used to spike the food and water matrices were obtained from pure cultures, and therefore do not represent the true state of bacterial populations found on food

matrices in diagnostic settings (injured, stressed cells) (98). Furthermore, prior to pursuing a 4 hr enrichment, 6 hr enrichment studies were performed. These studies resulted in the recovery of target STEC cell counts that were too great to enumerate (data not shown). As a result, an extension of the enrichment time from 4 to 6 hrs could be recommended to allow more time for the resuscitation of injured or stressed cells found in diagnostic samples, and to accommodate isolates that may be slow growers under enrichment conditions. In addition to capture, the experiments were designed to investigate the potential to visualize captured and eluted target cells on 0.22 μm black polycarbonate membranes. One of the major obstacles reported in literature is the interference of background microflora and matrix components during visualization following filtration (171). In the IFM procedure described in this thesis, the visualization of captured cells, was performed following IMS. This extra step following filtration aids in clarifying the sample to be visualized of most contaminating background microflora and matrix components. The results indicated that STEC target cells were easily detectable following a 4 hr enrichment (≥ 100 CFU) of a spiked water or juice sample (≤ 15 CFU). The method described could be further streamlined by conjugating the monoclonal antibodies to fluorescent tags. Once visualized, the bacteria can be easily recovered from the membrane and plated on selective/ differential media. Another advantage to the method described for the isolation of STEC in this thesis, is the use of an acidic elution step preceding IFM. The acidic elution buffer could help improve recovery of STEC on selective/ differential media (eg Rainbow agar) by inhibiting background microflora. This has been evidenced by a study that reported an improved detection of STEC O157 from alfalfa sprouts by subjecting captured cells to an acidic solution (pH 2) for 7 min following IMS (190). Though a major limitation of the method described in chapter 2 is its inability to be applied to more complex matrices; cross flow filtration could be a

potential filtration alternative to deal with high particulate matrices. This was evidenced by the recovery of *Salmonella* Enteritidis from chicken homogenates in under an hour using cross flow filtration (191). A similar method could be implemented for our purposes, followed up by IMS using monoclonal antibodies against the O antigen of target STEC, and IFM for the detection of STEC. Such a system will have to be tested with complex food matrices to assess its applicability in diagnostic testing.

The third chapter of this thesis focussed on the development of a flow through system that utilized large beads ($\geq 100 \mu\text{m}$) coated with monoclonal antibodies specific to the target STEC, packed into a column. The results obtained from these proof of concept experiments indicated that covalently coupled protein G polystyrene beads ($100 \mu\text{m}$) may serve as a promising candidate for the immobilization of highly specific monoclonal antibodies specific to STEC, which can then be used for the capture of these target cells from enrichment cultures. The method used to covalently conjugate protein G to the polystyrene beads was adapted from the procedure described by Bora *et al.* This group reported that the photoactivation of polystyrene plates using FNAB gave rise to ELISA values that were 3 times higher than the values obtained from traditionally coated ELISA plates (192). Our results also suggested that the photoactivation of polystyrene beads increases the protein G content on the bead when compared to passively adsorbed beads. The capture results indicated that the enrichment media components have an inhibitory effect on capture rates observed. The protein G conjugated polystyrene beads were only able to capture ~ 14 CFU of the expected 10^3 CFU of O157 cells from 250 ml of enrichment culture of a spiked water sample under the conditions used here. However, ~ 92 CFU of the target was captured of the 10^3 CFU of O157 cells spiked into 250 ml of diluted enrichment media (1:1 v/v in PBS). This indicated that the media components inhibit the interactions

between the antibody and the target; however these inhibitory effects could be diminished by diluting the enrichment media in PBS. These results are similar to a study that reported diminished ELISA values caused by interference from cell culture media components, which was rescued with the dilution of the culture medium (193). Since larger beads are used in the procedures described in chapter 3, and the column is fitted with a large pore size frit (30 μm), it is possible to combine pre-filtration steps used in chapter 2 for complex matrices and use the resulting filtrate in the procedure described in chapter 3. A combination of both these methods would have to be tested to determine its effectiveness and applicability in a diagnostic lab.

The current STEC diagnostic methods implemented at the CFIA rely heavily on attaining high bacterial densities following lengthy enrichment procedures. Enrichment is then followed by plating of diluted enrichment culture on differential/selective media with no prior isolation (MFLP-52), or IMS of a sample containing high concentrations of background microflora followed by plating on differential/ selective media (MFHPB-10). A presumptive positive test cannot be obtained in less than two days using either of the two methods. Furthermore confirming a presumptive positive can take an additional 2-3 days. The time consuming nature of these procedures can lead to a back log in diagnostic laboratories, and in some rare cases it could pose a threat to public health, as evidenced by the case of contaminated meat being recalled past its best before date in British Columbia (2014) (164). The two approaches described in this thesis offer a procedure that utilizes a short enrichment step (4-6 hrs), followed by pathogen capture using immobilized monoclonal antibodies specific to the target. Recovery in both these cases can then be detected by IFM which could provide diagnostic laboratories a means to have presumptive results within the first working day. Following a presumptive positive, the cells can then be easily recovered from the membrane and plated on differential/ selective media for

further characterization. Another option available to diagnostic laboratories is to use the recovered cells to perform PCR (targeting *eae*, *hlyA*, *stx1*, *stx2*, and *wzx* genes) based identification of the suspect organism as is done in the case of MFLP-52. As a result the procedures described in this thesis could provide diagnostic laboratories with means to obtain a presumptive positive and confirmatory result within the first 2 days of diagnostic testing when testing water and simpler food matrices.

4.2 CONCLUSIONS AND FUTURE DIRECTION

The work presented in this thesis provides two procedures that allows for the isolation of ~ 100 CFU of clinically significant STEC from the entire enrichment culture volume following a 4 hr enrichment of water spiked with ≤ 15 CFU of the target STEC. Similar results were obtained in the filtration model when apple juice was used as a food matrix and in the presence of competing microflora. In the flow through model, it was observed that capture rates were influenced by media components and the degree of contact afforded by the flow rate of the system. Both methods offer streamlined procedures to test for and confirm the presence of clinically significant STEC in water samples within 2 days of testing. The same can be said for clear juice samples when the filtration model is used.

Future work should be focussed on: (1) determining whether capture rates for covalently coupled protein G polystyrene beads could be improved by slowing down flow rates for large sample volumes, thereby increasing contact between the cells and the antibody, (2) determining whether capture by covalently coupled protein G polystyrene beads is hindered by enrichment cultures of simple and complex matrices, and if so determining if dilution can help restore capture rates, and (3) determining if filtering enrichment cultures of more complex matrices through a gradient

CHAPTER 4: General discussion and conclusion

of membranes (180- 41 μm) could then allow for capture of target cells from the filtrate when it is applied to the flow through model.

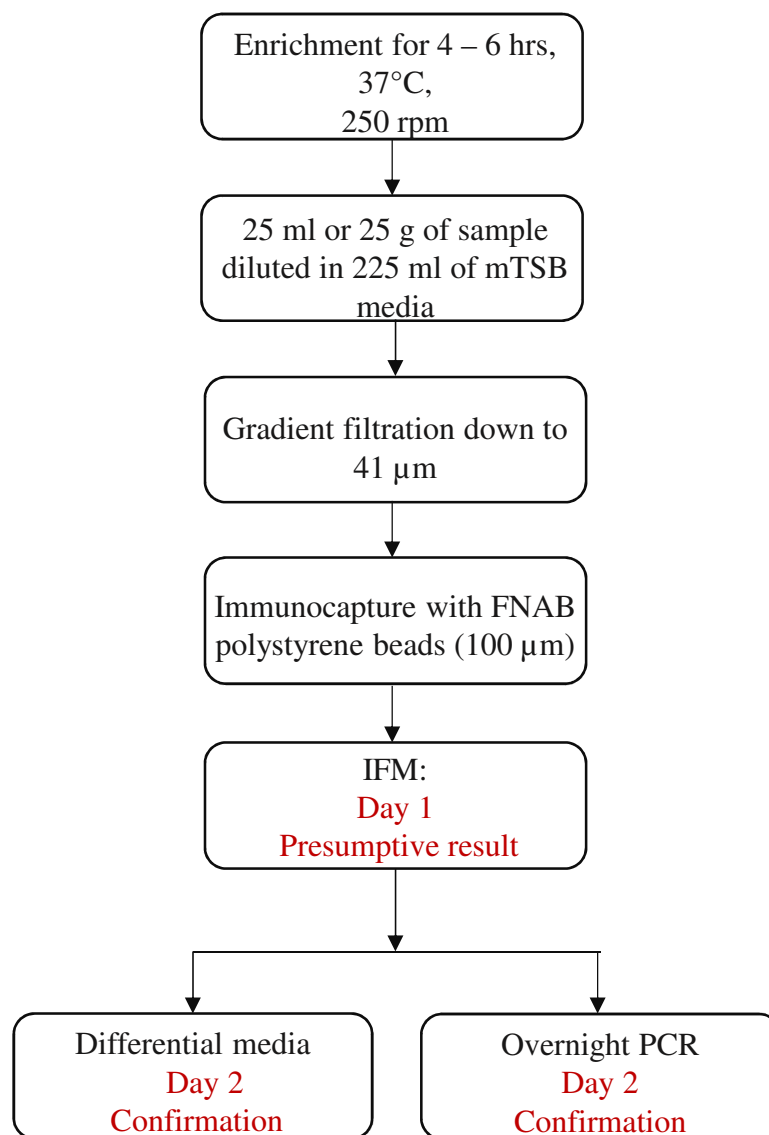


Fig 25: Potential workflow for the use of a combination of the gradient filtration and the flow through capture using large non-magnetic beads. The proposed method could potentially be implemented for both complex and simple food matrices. All matrices are diluted in non-selective media (mTSB) after which the samples are enriched for 4-6 hrs. Following enrichment, the samples are filtered through a series of filtration membranes with decreasing pore sizes (min. 40 μm) to remove larger matrix components. The filtrate is then added to a column containing covalently conjugated protein G polystyrene beads (100 μm), coated with mAb specific to the target STEC allowing capture of the target cells. Following capture, the eluted cells can then be visualized using IFM thereby providing a presumptive result within the first day of testing. The visualized cells can then be recovered and plated on differential media for further characterization. Another option available is to test the recovered cells using pre-established PCR protocols (MFLP-52) thereby simultaneously confirming the presence of STEC and its identity. Implementation of these protocols could in effect provide a confirmatory result within the first two days of testing thereby drastically streamlining the currently used procedures.

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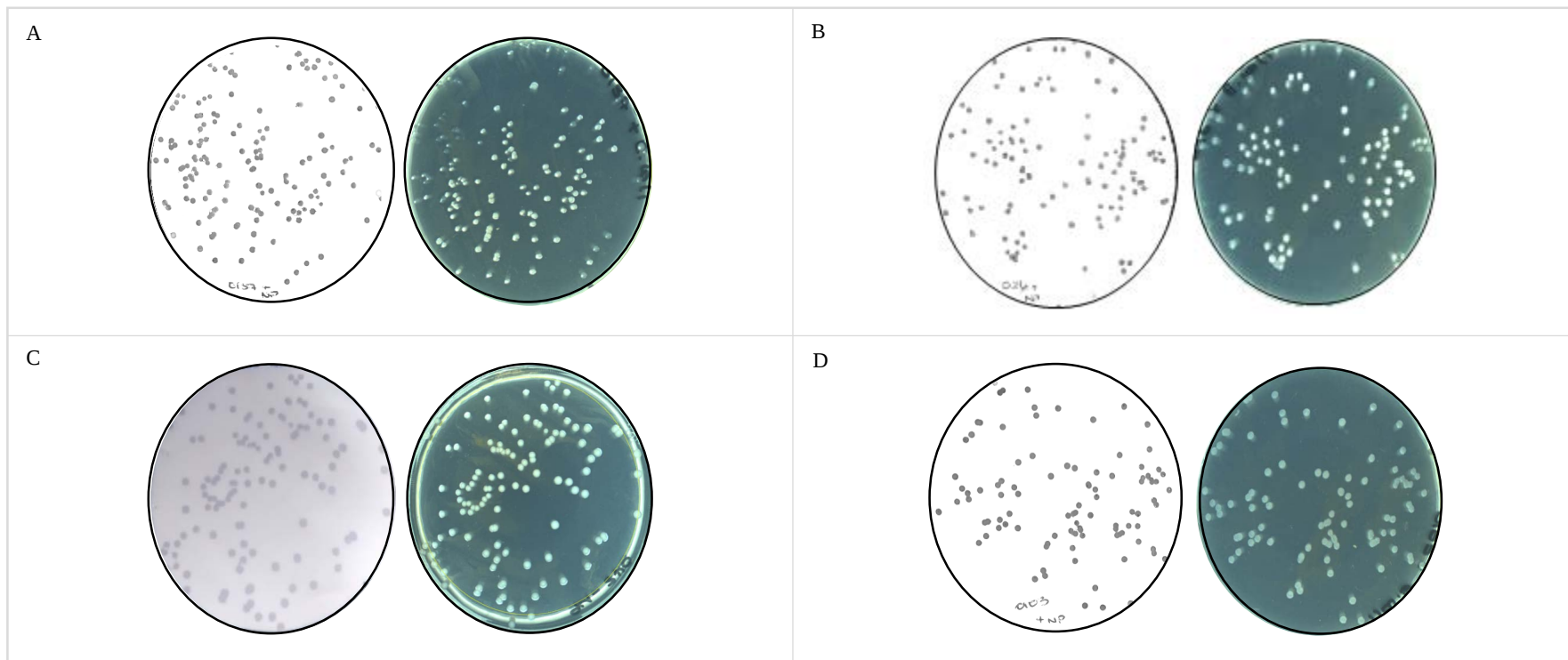


Fig A1.1 Colony blots resulting from specificity capture studies for target STEC in the presence of an excess of an isolate of non-pathogenic *E. coli* isolate as a competitor. Capture was performed using Dynal beads coated with monoclonal antibodies against the O antigen of the target. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. Membranes were probed with monoclonal antibodies specific to the O antigen of the target. The target serotype in panel A is O157, B is O26, C is O45, and D is O103.

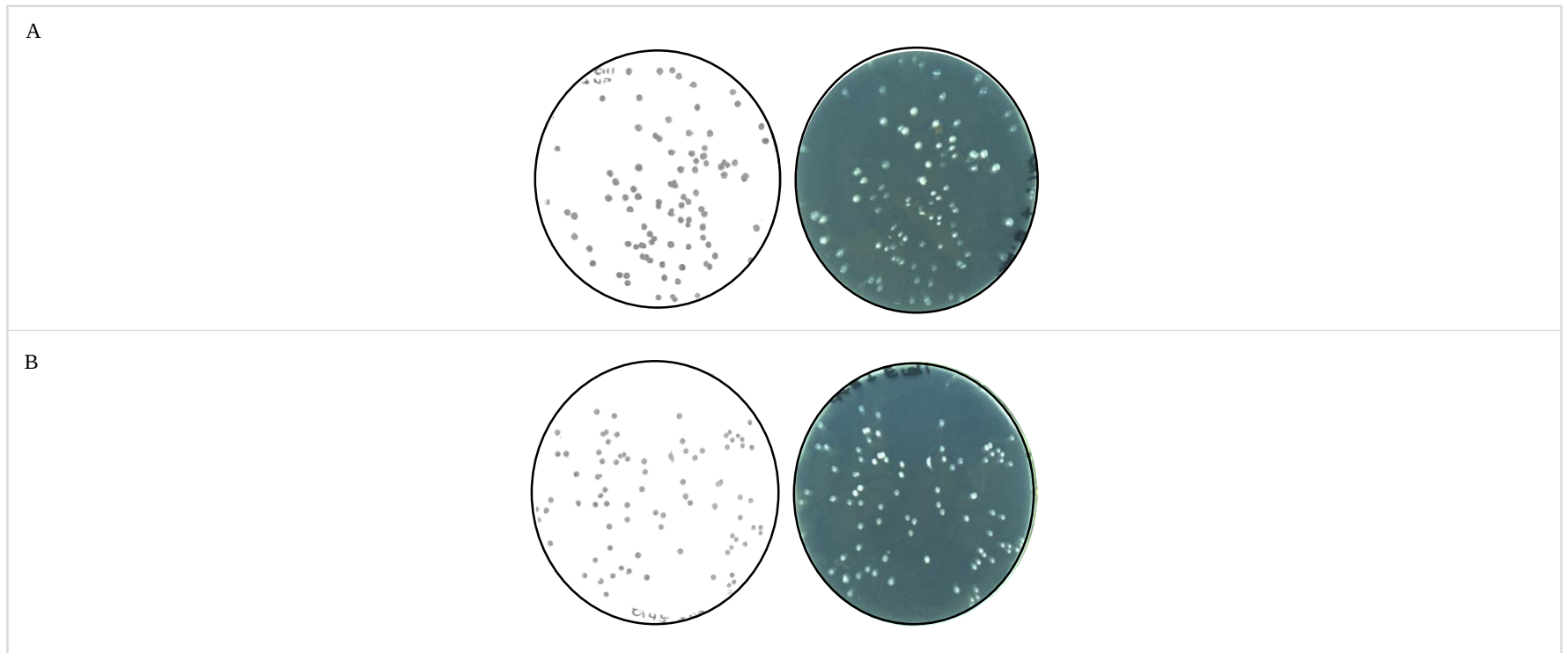


Fig A1.2 Colony blots resulting from specificity capture studies for target STEC in the presence of an excess of an isolate of non-pathogenic *E. coli* isolate as a competitor. Capture was performed using Dynal beads coated with monoclonal antibodies against the O antigen of the target. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. Membranes were probed with monoclonal antibodies specific to the O antigen of the target. The target serotype in panel A is O111, B is O145.

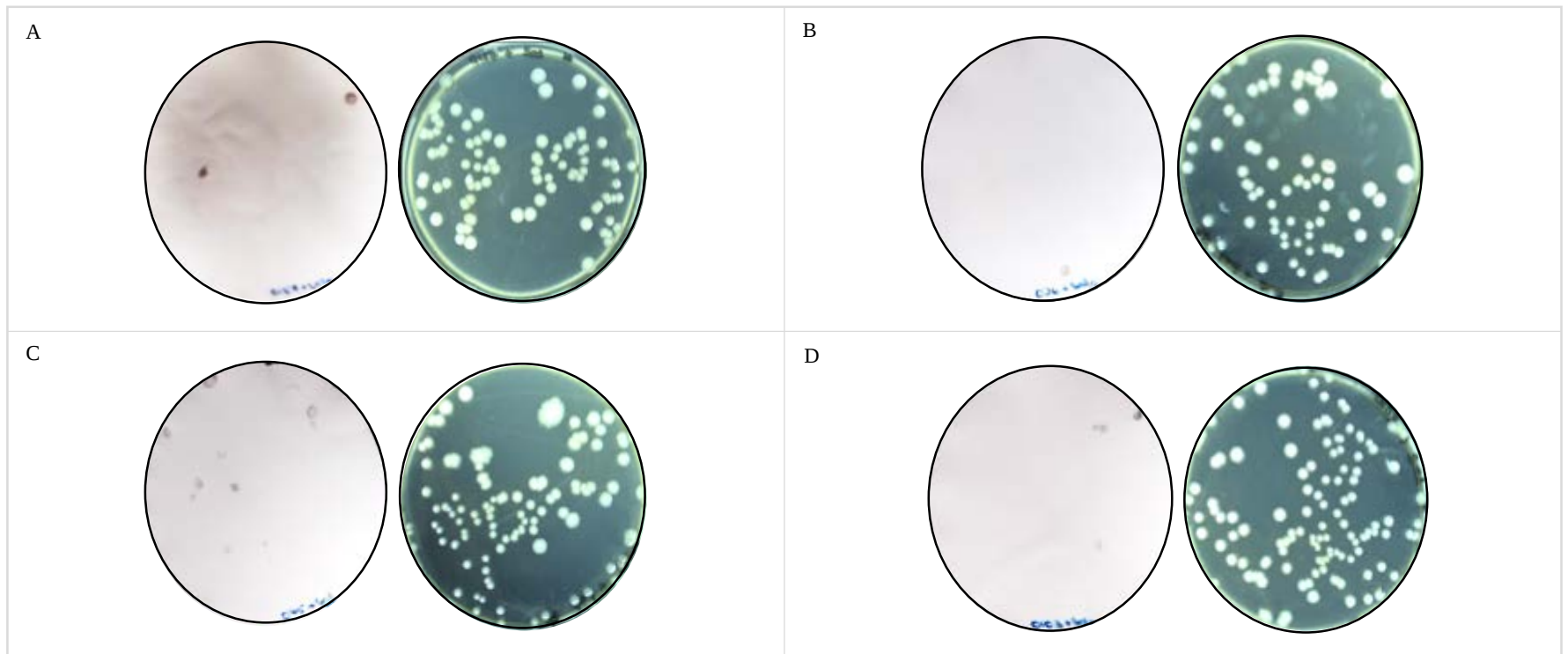


Fig A1.3: Colony blots resulting from specificity capture studies for target STEC in the presence of an excess of a *Salmonella* Typhimurium isolate as a competitor. Capture was performed using Dynal beads coated with monoclonal antibodies against the O antigen of the target. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. Membranes were probed with monoclonal antibodies specific to the *Salmonella* Typhimurium. The target serotype in panel A is O157, B is O26, C is O45, and D is O103.

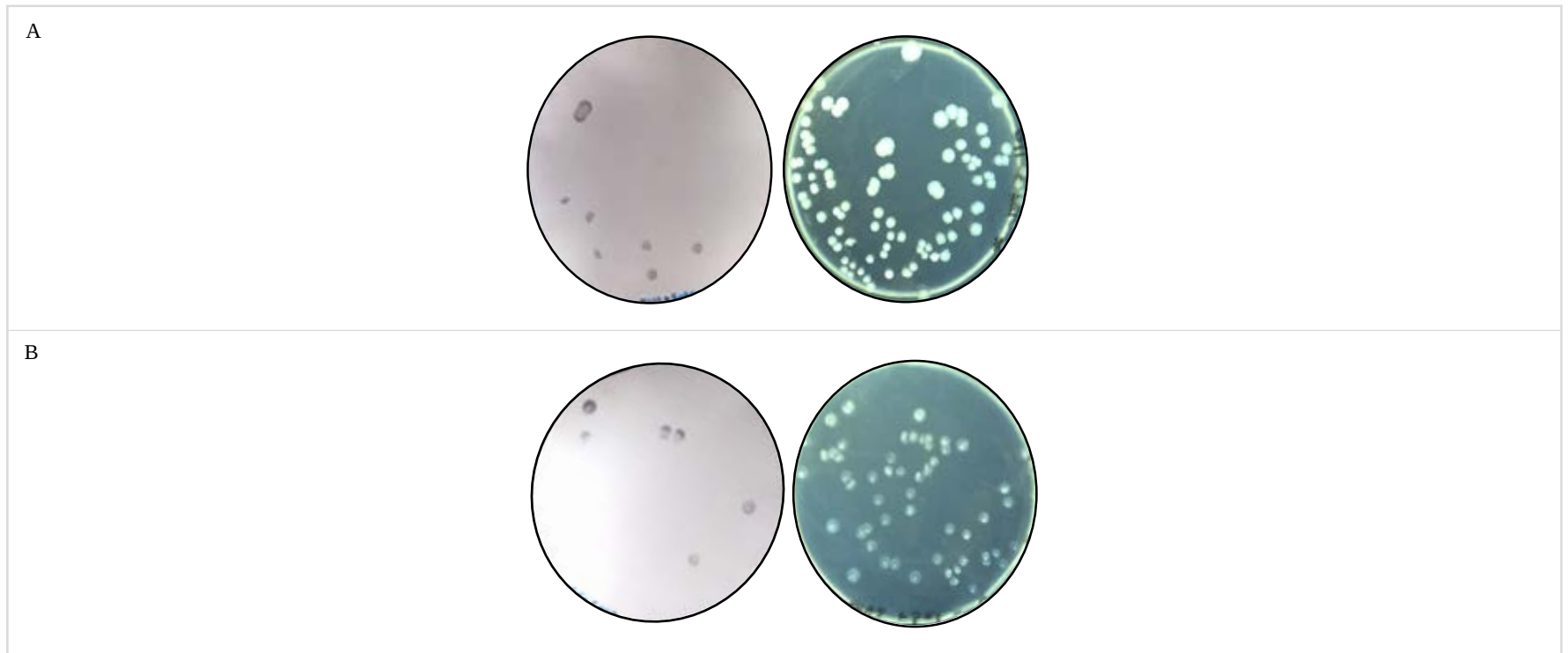


Fig A1.4: Colony blots resulting from specificity capture studies for target STEC in the presence of an excess of a *Salmonella* Typhimurium isolate as a competitor. Capture was performed using Dynal beads coated with monoclonal antibodies against the O antigen of the target. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. Membranes were probed with monoclonal antibodies specific to the *Salmonella* Typhimurium. The target serotype in panel A is O111, B is O145.

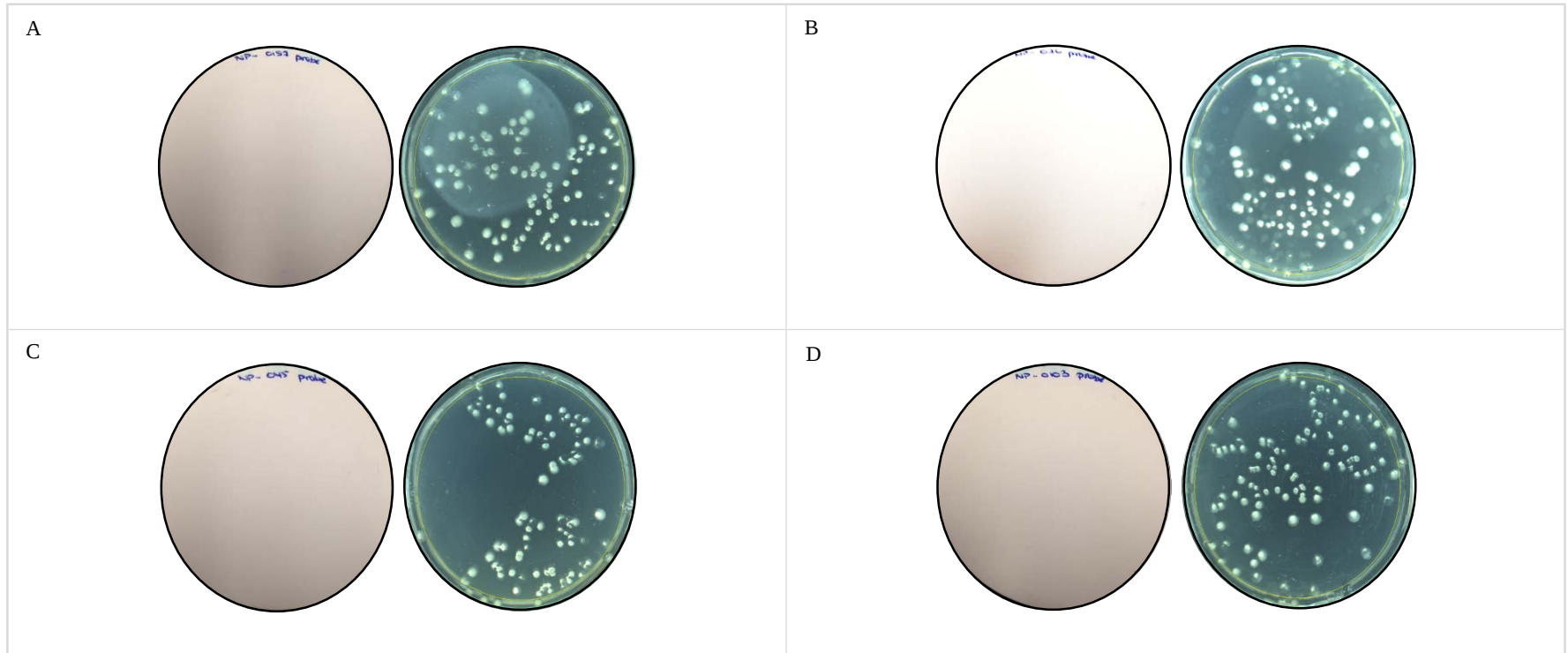


Fig A1.5: Control blots have been represented where each panel represents a colony blot and the corresponding bacterial plate containing only the non-pathogenic *E. coli* isolate. Membranes were probed with monoclonal antibodies specific to the O antigen of the STEC targets used in the specificity study. Specifically the membrane in panel A was probed with monoclonal antibodies against O157, panel B with monoclonal antibodies against O26, panel C with monoclonal antibodies against O45, and panel D with monoclonal antibodies against O103.

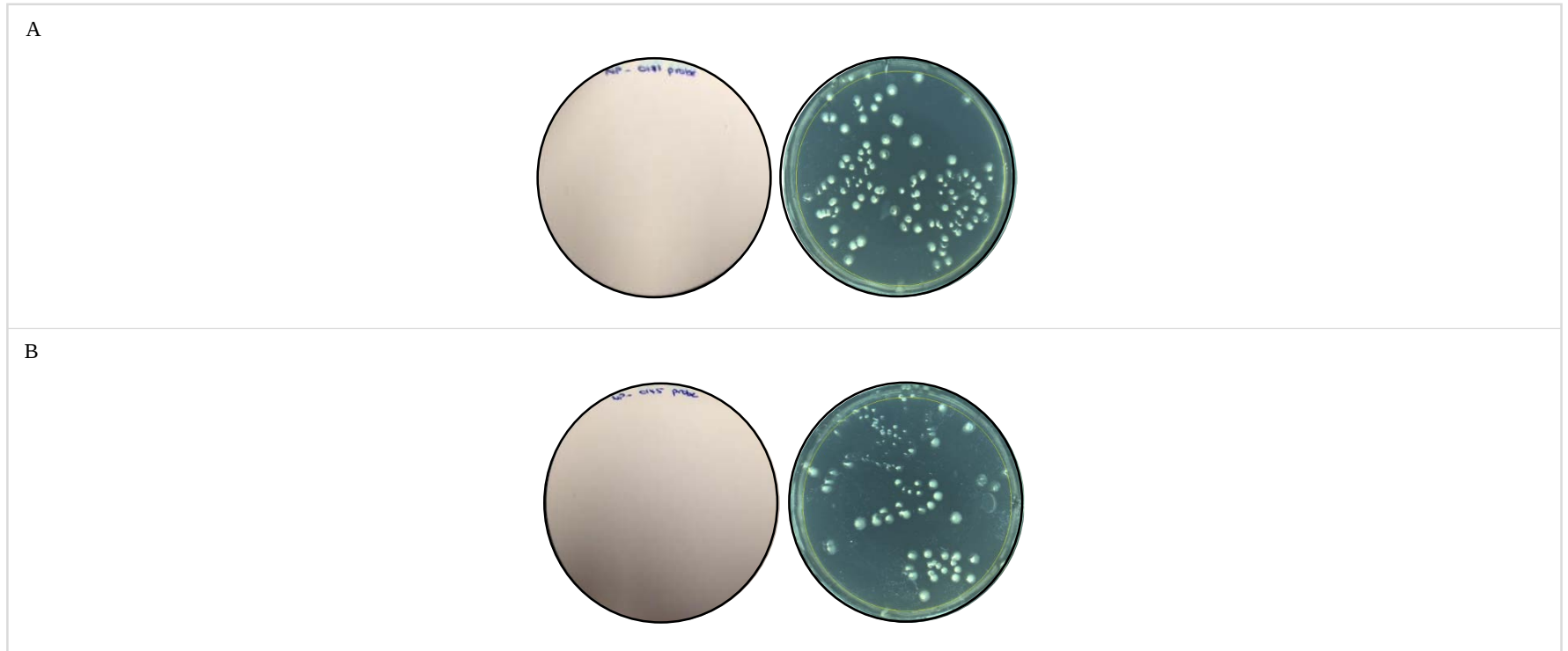


Fig A1.6: Control blots have been represented where each panel represents a colony blot and the corresponding bacterial plate containing only the non-pathogenic *E. coli* isolate. Membranes were probed with monoclonal antibodies specific to the O antigen of the STEC targets used in the specificity study. Specifically the membrane in panel A was probed with monoclonal antibodies against O111, panel B with monoclonal antibodies against O145.

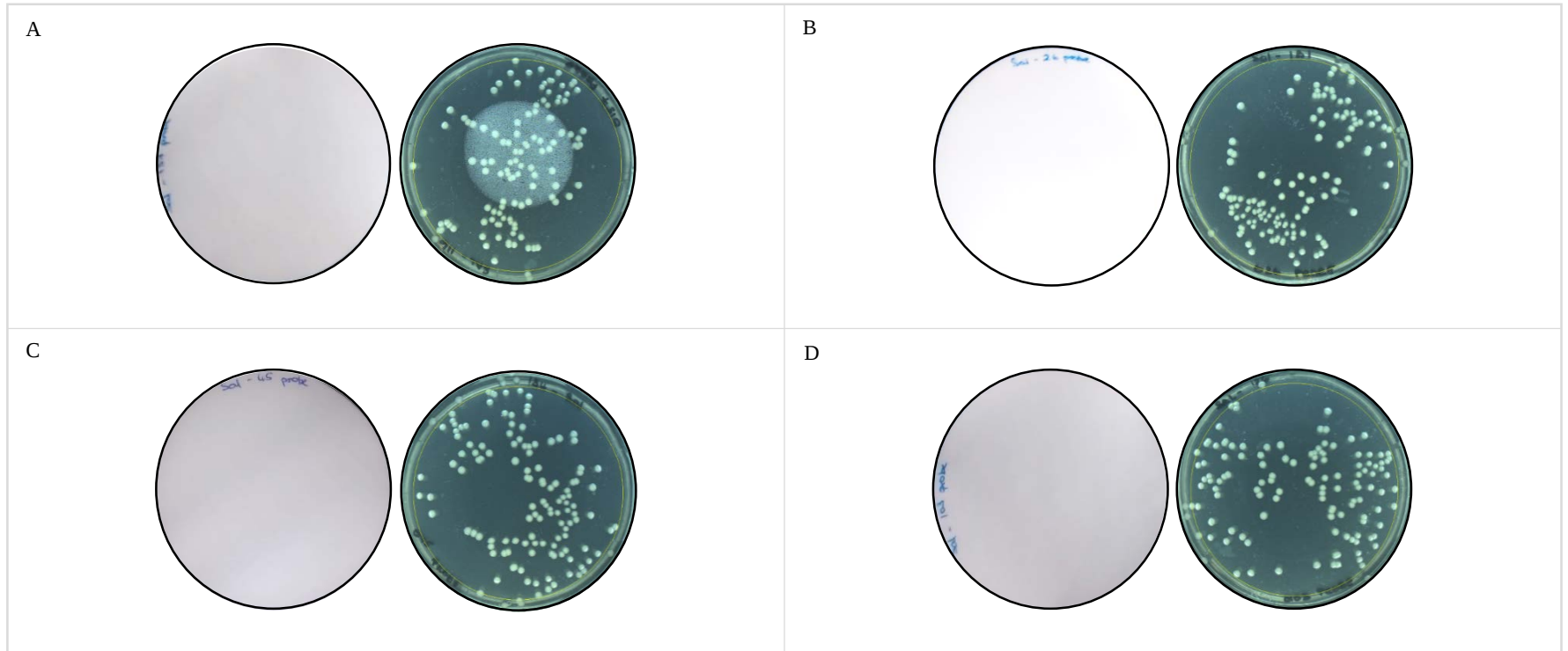


Fig A1.7: Control blots have been represented where each panel represents a colony blot and the corresponding bacterial plate containing only an isolate of *Salmonella* Typhimurium. Membranes were probed with monoclonal antibodies specific to the O antigen of the STEC targets used in the specificity study. Specifically the membrane in panel A was probed with monoclonal antibodies against O157, panel B with monoclonal antibodies against O26, panel C with monoclonal antibodies against O45, and panel D with monoclonal antibodies against O103.

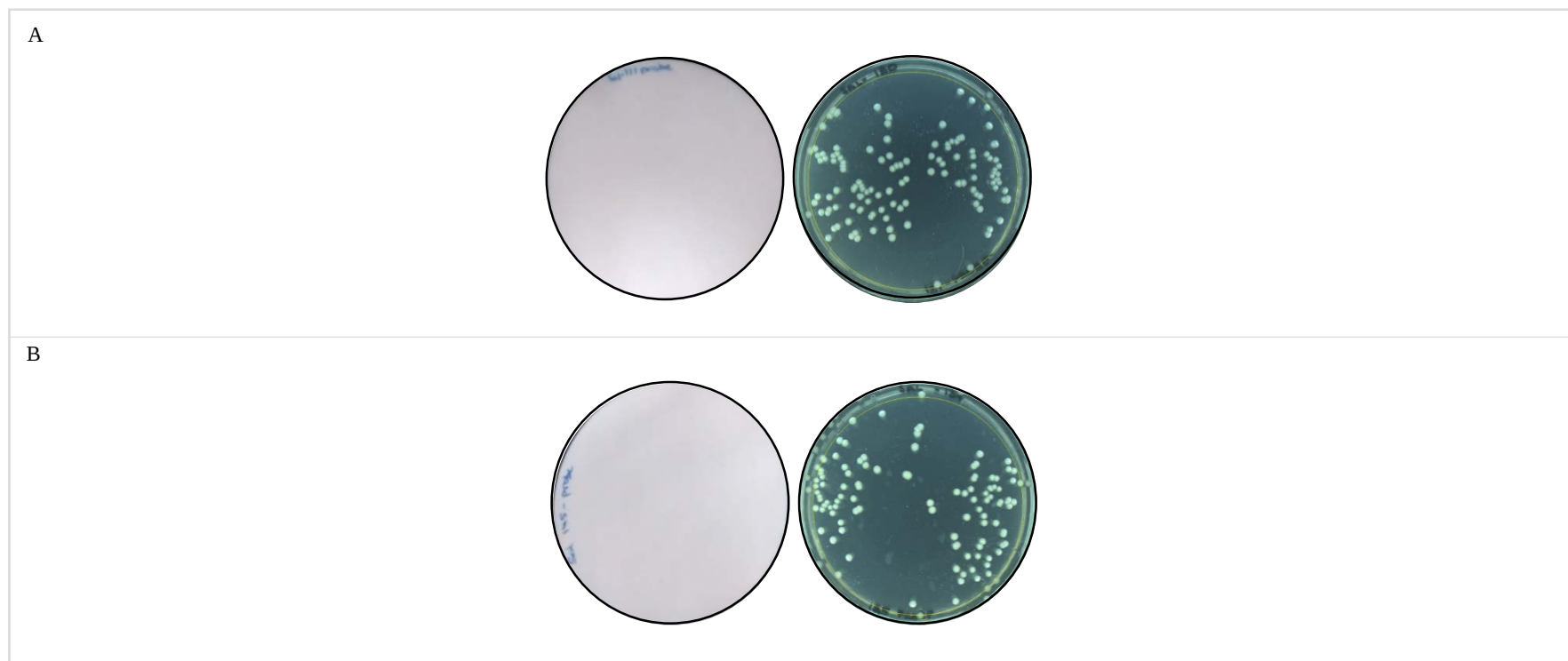


Fig A1.8: Control blots have been represented where each panel represents a colony blot and the corresponding bacterial plate containing only an isolate of *Salmonella* Typhimurium. Membranes were probed with monoclonal antibodies specific to the O antigen of the STEC targets used in the specificity study. Specifically the membrane in panel A was probed with monoclonal antibodies against O111, panel B with monoclonal antibodies against O145.

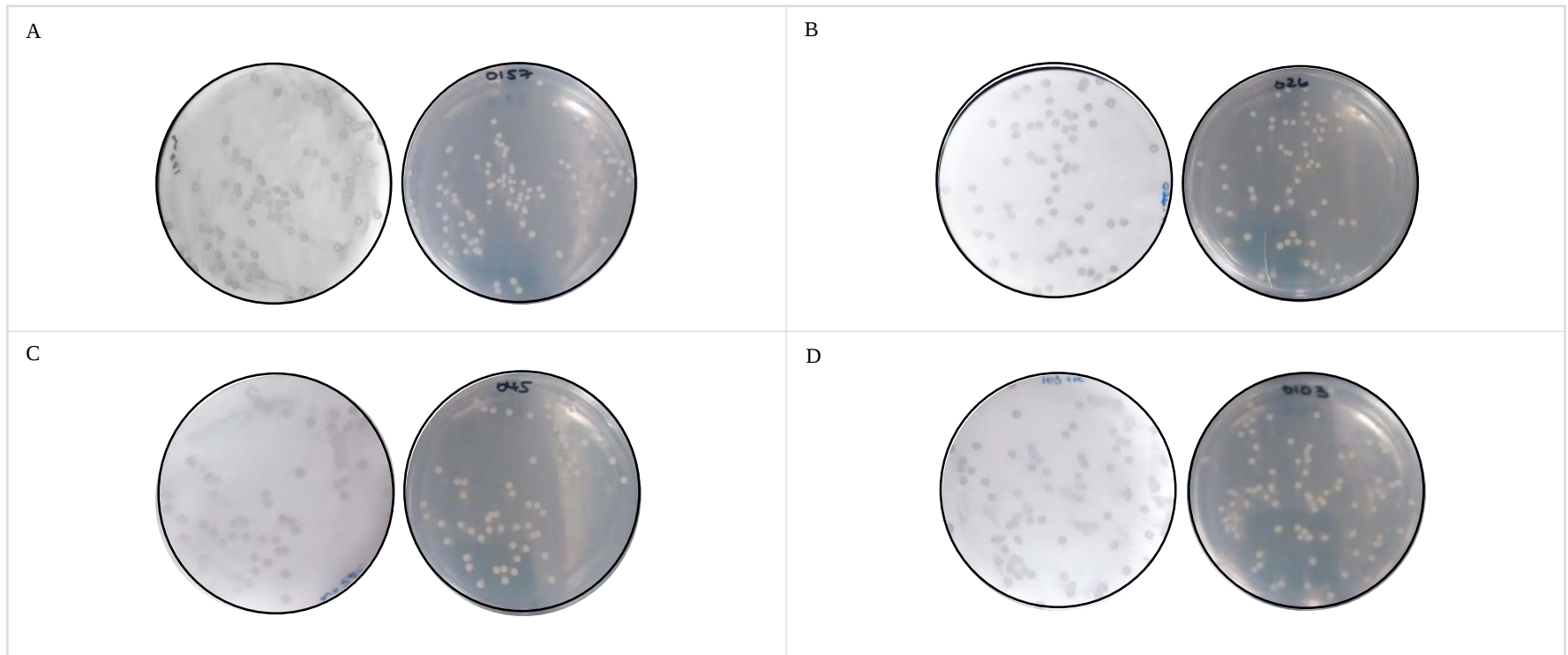


Fig A1.9: Control blots have been represented where each panel represents a colony blot and the corresponding bacterial plate containing only a target STEC isolate. Membranes were probed with monoclonal antibodies specific to the target. Specifically the membrane in panel A was probed with monoclonal antibodies against O157, panel B with monoclonal antibodies against O26, panel C with monoclonal antibodies against O45, and panel D with monoclonal antibodies against O103.

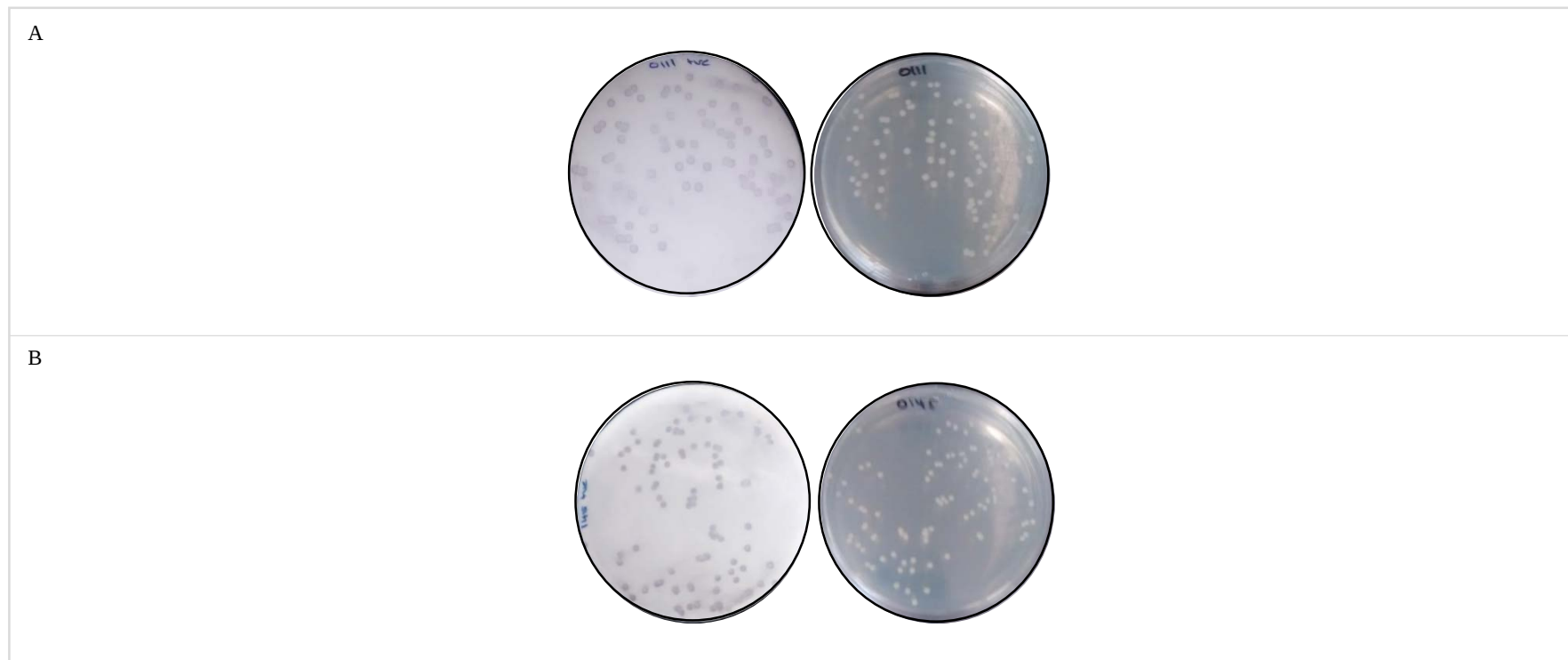


Fig A1.10: Control blots have been represented where each panel represents a colony blot and the corresponding bacterial plate containing only a target STEC isolate. Specifically, the membrane in panel A was probed with monoclonal antibodies against O111, panel B with monoclonal antibodies against O145.

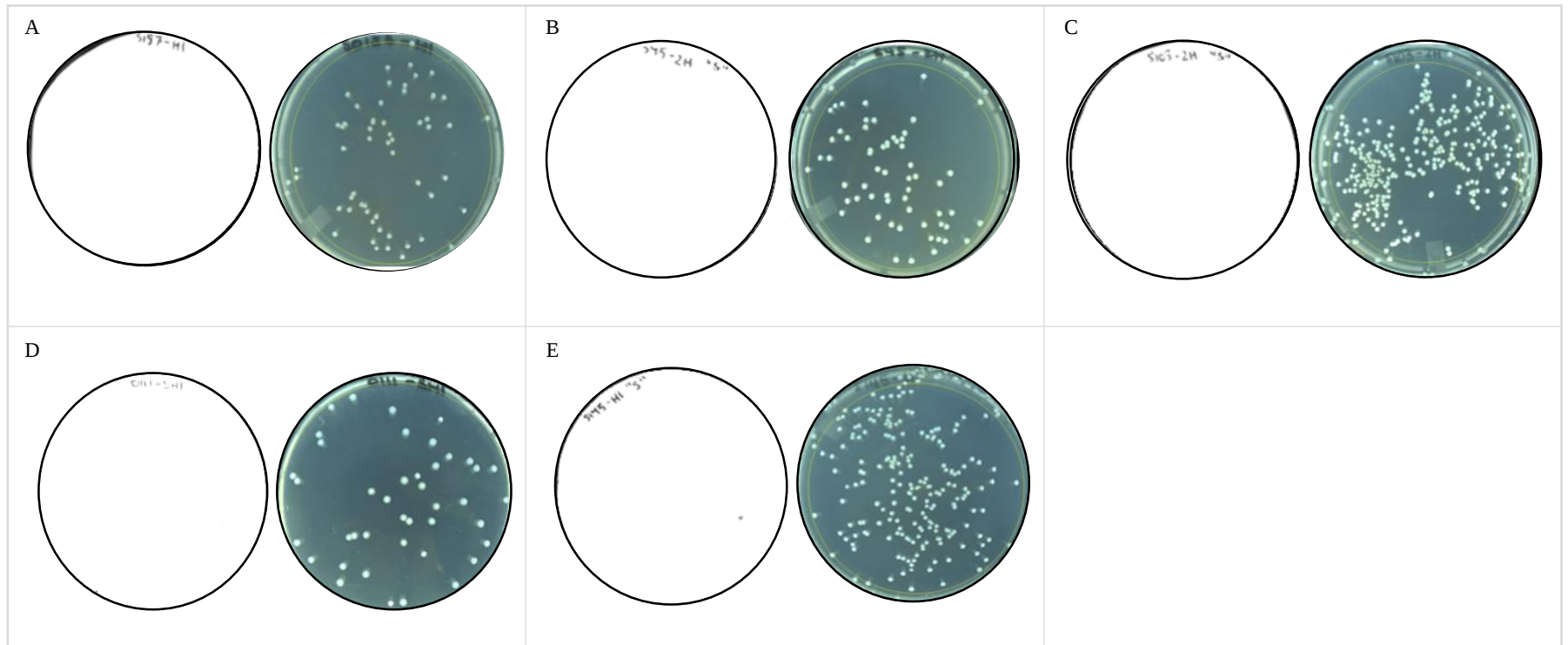


Fig A2.1: Colony blots resulting from capture studies performed following enrichment of a water sample spiked with both a target STEC isolate and an isolate of *Salmonella* Typhimurium as a competitor. Capture was performed using Dynal beads coated with monoclonal antibodies against the O antigen of the target. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. Membranes were probed with monoclonal antibodies specific to *Salmonella* Typhimurium. The target serotype in panel A is O157, B is O45, C is O103, D is O111, and E is O145.

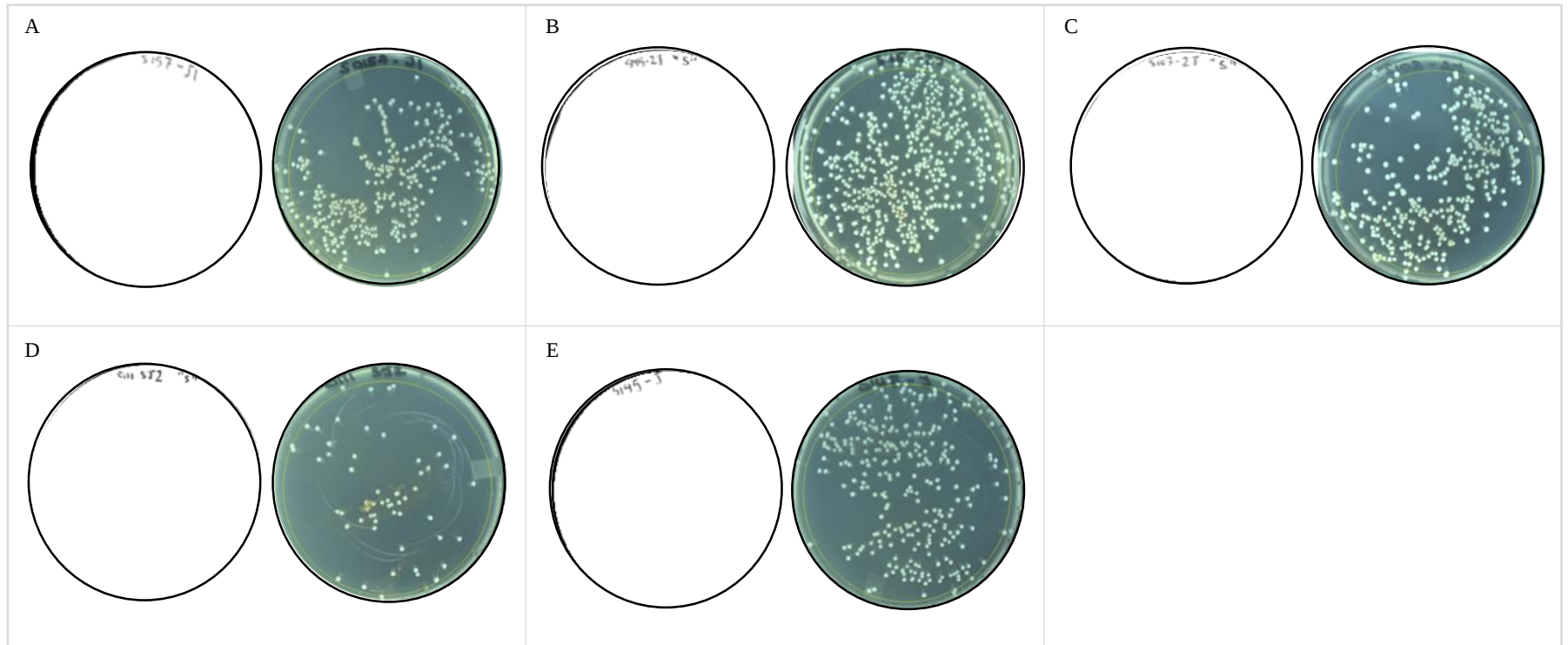


Fig A2.2: Colony blots resulting from capture studies performed following enrichment of a apple juice sample spiked with both a target STEC isolate and an isolate of *Salmonella* Typhimurium as a competitor. Capture was performed using Dynal beads coated with monoclonal antibodies against the O antigen of the target. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. Membranes were probed with monoclonal antibodies specific to *Salmonella* Typhimurium. The target serotype in panel A is O157, B is O45, C is O103, D is O111, and E is O145.

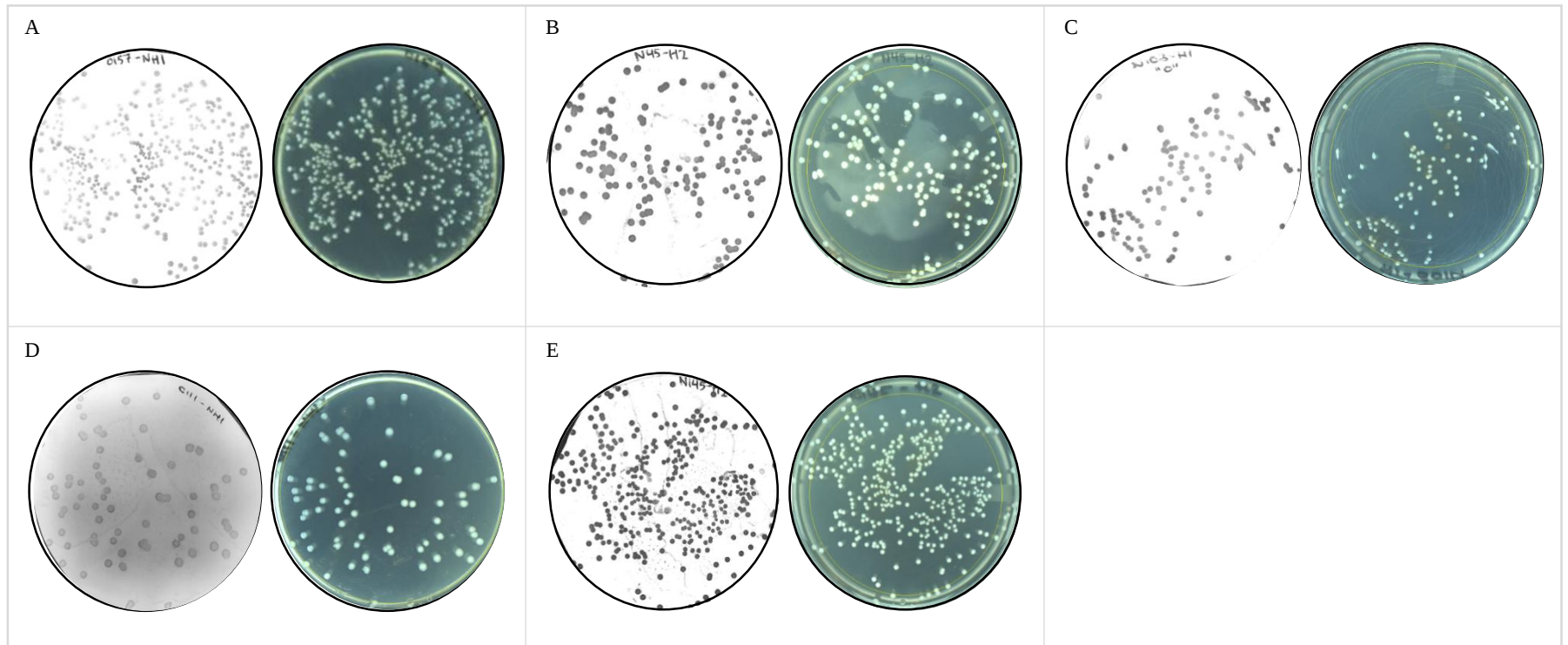


Fig A2.3: Colony blots resulting from capture studies performed following enrichment of a water sample spiked with both a target STEC isolate and an isolate of non-pathogenic *E. coli* as a competitor. Capture was performed using Dynal beads coated with monoclonal antibodies against the O antigen of the target. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. Membranes were probed with monoclonal antibodies specific to the target STEC. The target serotype in panel A is O157, B is O45, C is O103, D is O111, and E is O145.

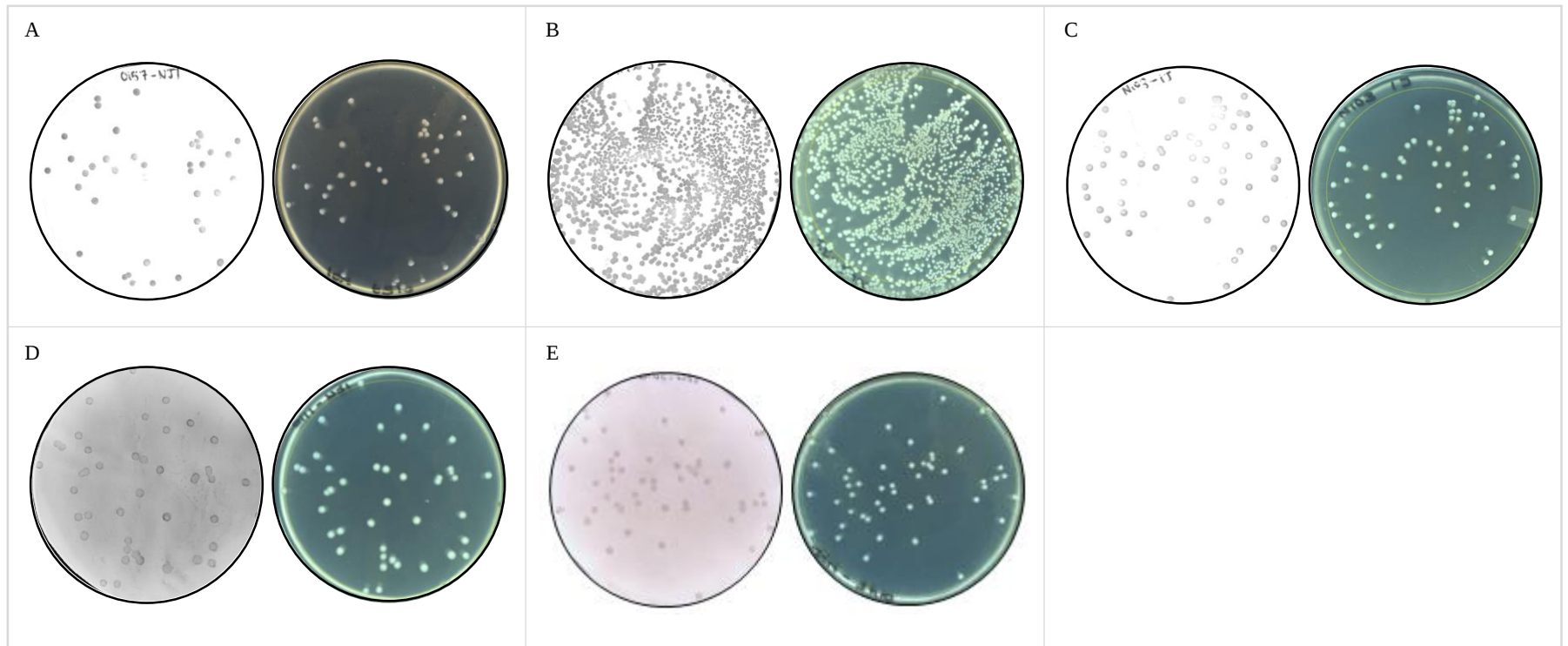


Fig A2.4: Colony blots resulting from capture studies performed following enrichment of an apple juice sample spiked with both a target STEC isolate and an isolate of non-pathogenic *E. coli* as a competitor. Capture was performed using Dynal beads coated with monoclonal antibodies against the O antigen of the target. Each panel represents a colony blot and the corresponding bacterial plate containing captured bacteria. Membranes were probed with monoclonal antibodies specific to the target STEC. The target serotype in panel A is O157, B is O45, C is O103, D is O111, and E is O145

CONTRIBUTION OF COLLABORATORS

Dr. Brian Brooks produced and provided all the monoclonal antibodies against the LPS O antigen of target STEC (O157, O26, O45, O103, O111, O121, and O145).

Hanhong Dan provided the vector pPG containing the recombinant protein G gene for the work performed in Chapter 3