THESIS

EVALUATION OF GENE-UP AND TEMPO AC FOR DETERMINATION OF SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* AND TOTAL AEROBIC MICROBIAL POPULATIONS FROM MICROTALLY SHEETS USED TO SAMPLE BEEF CARCASSES AND HIDES

Submitted by

Tianqing Liu

Department of Animal Sciences

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Master's Committee:

Advisor: Keith E. Belk
Co-Advisor: Hua Yang

Francisco J. Zagmutt
Tiffany L. Weir

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ABSTRACT

EVALUATION OF GENE-UP SYSTEM AND TEMPO AC TEST FOR DETERMINATION OF SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* AND TOTAL AEROBIC MICROBIAL POPULATIONS FROM MICROTALLY SHEETS USED TO SAMPLE BEEF CARCASSES AND HIDES

Two studies were conducted to evaluate GENE-UP and TEMPO AC (bioMerieux, Marcy-l'Étoile, France) for determination of Shiga-Toxin producing *Escherichia coli* and total aerobic microbial populations from MicroTally Sheets (Fremonta Corporation, Fremont, CA) used to sample beef carcasses and hides. The first study was conducted to evaluate the automated TEMPO® AC Test in comparison with traditional direct agar plating method for enumeration of aerobic mesophilic flora in MicroTally sheets used to sample beef carcasses and hides. A total of 160 MicroTally (MT) sheet samples were collected from commercial beef processing plants by swab-sampling on the surface of naturally contaminated pre-evisceration carcasses, hides and post-chill final carcasses, and analyzed within 24 h after sample collection. Of these, all 160 samples were within detection limit and analyzed by both automated TEMPO AC test and a traditional direct agar plating method. For these results, the aerobic count correlation coefficient was high (0.93) for pre-evisceration carcasses, which had mean (± standard deviation) counts of 3.3 ± 0.9 and 3.1 ± 0.8 log CFU/mL for those two methods, respectively. The aerobic count correlation coefficients were higher (0.95 and 0.96) for MT samples from hides and post-chill final carcasses, which had mean (± standard deviation) counts of 5.3 ± 1.2 and 5.0 ± 1.2, 3.0 ±

1.4 and 3.0 ± 1.3 log CFU/mL for those two methods, respectively. Overall, 98.8% of aerobic count results were within 1.0-log difference between the two enumeration methods. The correlation coefficient (r = 0.97) and linearity regression (log TEMPO MPN/mL = 1.06 x log PCA-CFU/mL +0.03) between the two methods was calculated for our whole sample set (n = 160). Our results demonstrated that the automated MPN method-TEMPO AC Test generated total aerobic mesophilic microflora counts that were highly correlated and consistent with the counts obtained by traditional plating methods on enumerating total aerobic mesophilic microbial populations recovered from MicroTally sheets. Use of TEMPO AC test for MicroTally sheet analysis could save time and labor for the meat industry as it conducts microbial analyses.

The second study was conducted to determine the specificity of bioMérieux's GENE-UP, a PCR-based molecular diagnostic system, to detect Shiga Toxin-producing *Escherichia coli* (STEC) from samples collected from beef processing plants using MicroTally sheets with the manual sampling device method. A total of 194 MicroTally (MT) samples were collected from beef processing plants and analyzed for determination of the top 6 STEC and *E. coli* O157: H7 (top 7 STEC) using the GENE-UP system, BioRad commercial kits and BioControl GDS kits. Fifty MT samples were collected from swabbing pre-evisceration carcasses and inoculated with hide-derived inocula, while the remaining 144 MT samples were obtained from post-chill final carcasses in sales coolers and inoculated with *E. coli* strains. All inoculated MT samples were enriched for 8-hour and 10-hour at 42°C in buffered peptone water (BPW) and re-collected after incubation. Eight-hour and 10-hour enrichment samples were analyzed using the GENE-UP system at Colorado State University and sent to U.S Meat Animal Research Center (USMARC, Clay Center, NE) for detection of top 6 STEC and *E. coli* O157: H7. The GENE-UP system uses

EH1 assay to detect *stx* and *eae* genes, ECO assay to detect genes specific to O157:H7 serogroup, and EH2 assay to differentiate top 6 serogroups. These virulence genes including Shiga-toxin gene (*stx*), intimin-encoding *eae* gene and genes specific to top 7 serogroups are highly related to pathogenic STEC. The NM-EHEC assay targeting virulence genes *espK, espV* and *CRISPR_O26E* does not directly differentiate the top 7 STEC, but serves as additional screening test to help identify presence of any of the top 7 STEC. All potential positive samples determined by PCR screening were plated onto selective agar for culture confirmation. After the immunoconcentration step, isolates picked from selective agar were subjected to additional PCR screening. BioRad and BioControl GDS PCR screening methods were used following their standard protocols for determination of top 7 STEC at USMARC. Presumptive positive samples confirmed by the additional PCR test were designated as "true positives." Presumptive positive samples that were not confirmed by the additional PCR test were designated as "regulatory false positives." Overall, our results indicated that the GENE-UP system worked well in the detection of the top 7 STEC recovered from the MicroTally sheets. In order to reduce or eliminate false negative results, a 10-h enrichment time in BPW was required for detection of both the top 6 STEC and *E. coli* O157:H7. Compared to GENE-UP and GDS, BioRad generated a much higher number of potential positives that required cultural confirmation. Moreover, use of the NM-EHEC kit targeting virulence genes (*espK, espV* and *CRISPR_O26E*), as an additional PCR screening after EH1 PCR (*stx* and *eae*), has potential to reduce the number of samples that require further O-type determination. However, the GENE-UP *E. coli* O157:H7 detection system needs to reduce rates of false negative results caused by the shift of T_m when *E. coli* O157:H7 and O157: non-H7 co-exist in a sample.

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CHAPTER 1: LITERATURE REVIEW

Pathogen Contamination of Beef

Beef production and consumption have increased over the past 60 years. Pathogen contamination in processing plants is associated with food safety as carcasses are further processed into beef products (Stromberg et al., 2018). Since mid-1990s, importance has been recognized of reducing the risk of pathogens in U.S. beef products (Wheeler et al., 2014). Skeletal muscle from healthy animals are considered inherently sterile, except for the lymph nodes (Huffman et al., 2002). However, the extrinsic sources, such as fecal material, intestinal contents, and the hide can contribute to carcass and meat contamination when the carcass comes into contact with these contaminants (Huffman et al., 2002). In addition, many other sources, such as processing equipment, human contact, and carcass-to-carcass contact, also are considered as possible cross contamination factors in the slaughter process (Huffman et al., 2002). As a result, to reduce potential pathogen contamination in processing plants, pre- and post-harvest interventions have been applied in beef industry for pathogen control and reduction (Wheeler et al., 2014). Nowadays, several interventions (i.e., trimming, steam vacuuming, steam pasteurization, water washes, and organic acid washes) in combination are applied in many commercial beef processing plants with the goal of large reduction and minimization in carcass contamination (Arthur et al., 2004). *E. coli* O157:H7 (declared as “adulterant” in non-intact beef by USDA-FSIS), in addition to non-O157 STEC and *Salmonella* spp. are considered the greatest-concern pathogens of meat carcasses, therefore they are first controlled at the slaughter process (Huffman et al., 2002).

Many studies have shown that the hide is the primary source of carcass contamination and *E. coli* contamination occurs primarily during hide removal (Bosilevac et al., 2004; Bosilevac et al., 2005; Wheeler et al., 2014). The nature of the hide removal process makes it hard to avoid the transfer of contamination from the hide surface to the carcass due to direct hide-to-carcass contact or by indirect transfer such as worker's gloves or clothes (Mcevoy et al., 2000). An investigation conducted in three beef processing plants by Barkocy-Gallagher et al. (2003) reported that 60.6% of hide samples were O157-positive before the pre-evisceration wash, and the prevalence was high from spring through the fall. One way to reduce contamination (Barkocy -Gallagher et al., 2003) was described as removing the hide immediately before firm bacteria attachment to the meat surface. However, there is a high chance that transfer of pathogen occurs during transportation and lairage. Pathogen contamination can occur within lairage from animal-to-animal, animal-to-environment, and environment-to-animal routes (Small et al., 2002). Implementation of Hazard Analysis and Critical Control Point Systems (HACCP) and Good Management Practices (GMP) for pre-harvest control of contamination associated with transportation, sanitation practices, and cattle drinking water can help to reduce the level of both meat spoilage and pathogenic microorganisms (Eisel et al., 1997; Callaway, 2010). Therefore, a whole systematic approach such as HACCP is necessary in processing plant for pathogen control.

Food Safety Interventions in Beef Industry

The outbreak of *E. coli* O157:H7 in 1993 catalyzed reform of a series of regulations and policies for monitoring and controlling of pathogens in meat (Wheeler et al., 2014). Enforcement of zero tolerance for *E. coli* O157:H7 was implemented by FSIS right after the outbreak of *E. coli* O157:H7, and since then this pathogen is considered adulterant in non-intact beef cuts,

ground beef and trimmings. Later in 1996, new regulations published in the USDA FSIS Final Rule added the mandated Hazard Analysis and Critical Control Point (HACCP) as one of the four required establishment programs for meat and poultry processing plants. More recently, in 2011, six non-O157 (O26, O103, O111, O145, O45, O121) STEC serogroups were declared as adulterants in non-intact, trimmings, and ground beef products (Wheeler et al., 2014). Generally, all regulations are aimed at better monitoring and minimizing contamination of products with pathogenic micro-organisms to ensure safety of food supplies. To achieve this goal, pre- and post-harvest interventions are widely applied in the U.S. beef industry (Wheeler et al., 2014).

Antimicrobial interventions

Numerous antimicrobial interventions applied in the beef industry are aimed at reducing or eliminating pathogenic microorganisms. Moreover, most antimicrobial interventions are implemented predominantly in the beef industry to reduce prevalence of *E. coli* O157:H7 due to its adulterant classification by USDA-FSIS (Kalchayanand et al., 2012). Many antimicrobial solutions are applied in multiple hurdle systems such as acetic acid, citric acid, lactic acid, peroxyacetic acid, acidified sodium chlorite and so on. Gorman et al. (1995) revealed that application of hot water during beef processing can effectively reduce the pathogens (up to 3.0-log CFU/cm^2 of reduction). Their study also reported that chemical interventions (i.e., spray-washing solutions) assist with reduction of microbial counts when following spraying with water of lower temperatures (e.g., 35°C) (Gorman et al., 1995). A study conducted by Yoder et al. (2012) compared the bactericidal effectiveness of eight antimicrobial compounds at various concentrations with hand-held spraying equipment to reduce cocktail inoculant pathogens and natural microbial populations. As a result, organic acids was determined with the greatest bactericidal effectiveness, while aqueous ozone resulted less bacterial reduction.

In addition to application of chemical solutions, other antimicrobial interventions such as hot water washing can also facilitate pathogen control in the meat industry. The condition set for hot water rinse on bovine head is around 74 °C for 12 and 26 seconds in a commercial spray cabinet, and as a result, reduce by 2.99-log microbial loads can be achieved (BIFSCO, 2016). A study conducted by Barkocy-Gallagher et al. (2003) demonstrated the high efficacy of antimicrobial interventions to reduce prevalence of pathogenic STEC and *Salmonella* on commercial beef products. In the Barkocy-Gallagher et al. (2003) study, initial recovery of *Salmonella* ranged from 3% to 24.9% for samples collected immediately after hide removal and prior to any antimicrobial interventions. After a full complement of antimicrobial interventions, reduction on *Salmonella* prevalence was observed, and the end recovery rate for this pathogen was less than 0.3%.

Multiple hurdles for pathogen control

The "multiple hurdles" concept was developed to combine several factors to eliminate, prevent, and control pathogen growth on raw beef (Ariyapitipun et al., 2000; Sofos & Smith, 1998). The multiple hurdles decontamination approach is sometimes involved with several applications of treatment simultaneously, or the sequential application of treatments including, but not limited to, hide cleaning, steam vacuuming, pre-evisceration washing, hot water carcass washing and organic acid rinsing (Hui, 2005). Studies have been conducted to compare effectiveness of single decontamination process or multi-hurdle approach, and as a result, data have shown that the multiple-sequential decontamination interventions resulted much higher reduction on microbial populations than a single intervention (Bacon et al., 2000; Dias-Morse et al., 2014; Graves Delmore et al., 1997).

A study by Barkocy-Gallagher et al. (2003) reported that prevalence of non-O157:H7 STEC and *stx* - harboring cells decreased to 4.0% after several interventions applied during processing, for which the initial positive rates were 13.9%, 56.1%, and 64.9% for feces, hides and pre-evisceration carcasses, respectively. In the same study, researchers also measured prevalence of *E coli* O157:H7 at different processing point. As a result, prevalence of *E. coli* O157:H7 in postintervention carcasses was less than 1.0%, while the initial positive rates were 12.9%, 73.5% and 40.8% for feces, hides and pre-evisceration carcasses, respectively. Another study that tested prevalence of *E coli* O157:H7 at various steps in commercial beef plants also reported high effectiveness of current multiple interventions. (Arthur et al., 2014). In this study, 76% of animal hides that tested positive for *E coli* O157:H7 at the time of entry into plants while no carcasses leaving the cooler were identified as being contaminated with this pathogen. Overall, the current multiple-hurdle interventions are necessary and effective on pathogen control in beef plants.

Escherichia coli **O157:H7 and non-O157 STEC**

E. coli is a Gram-negative, rod-shaped, facultative anaerobe that lives in the gastrointestinal tract of animals in health and disease (Todar, 2008). Most *E. coli* are considered harmless to humans, however, certain pathogenic *E. coli* strains can cause severe foodborne illness through consumption of contaminated food or water (Swaggerty et al., 2018). Based on the pathotypes, pathogenic *E. coli* strains are classified into 6 basic classes: enterotoxigenic, enteropathogenic, enteroaggregative, enteroinvasive, diffusely adherent and enterohemorrhagic (CDC, 2020a; Nataro et al., 1998). Enterohemorrhagic *E.coli* (EHEC) serotypes, also known as Shiga toxin-producing *E.coli* (STEC) or verocytotoxin-producing (VTEC), cause symptoms ranging from mild diarrhea to hemorrhagic colitis and in serious cases,

hemolytic uremic syndrome (HUS) (Tarr et al., 2005; Goldwater et al., 2012). In addition, beef cattle are considered to be primary reservoirs of O157 and non-O157 STEC (Hussein, 2007; Hussein et al., 2005). According to Martin and Beutin, food producing animals, including cattle, are the most important sources for entry of STEC into the food chain. Contaminated beef and water are major sources of STEC infection (Kumar et al., 2004). Therefore, pathogenic STEC is regarded as one of the greatest concerns in raw, non-intact beef products.

The STEC serotypes are classified within the *Enterobacteriaceae* family and are named based on their somatic (O) and flagellar (H) surface antigen profiles (Nataro et al., 1998). Infections with STEC's are mostly foodborne. Presence of fewer than 1000 bacteria in contaminated food (e.g., undercooked ground beef, raw milk) can cause human infection (Ahn et al., 2008; Yang et al., 2017). Clinical symptoms, including watery diarrhea, HC, hemolytic uremic syndrome (HUS), fever, abdominal cramping, and vomiting, can be caused by STEC infections (Yang et al., 2017). In 1982, a STEC O157:H7 infection was linked to hemorrhagic colitis and the hemolytic-uremic syndrome (HUS). Since then, around 250 different O serogroups of *E. coli* have been shown to produce Shiga toxin, and more than 100 of these STEC have been associated with sporadic and epidemic human diarrheal diseases (James et al., 2006). Those certain serotypes frequently associated with outbreaks and severe clinical illness are within a subgroup of STEC and defined as EHEC. Among the EHEC group, pathogens including *E. coli* O157:H7 and the top six non-O157 STEC serogroups (O26, O45, O103, O111, O121, and O145), also referred to as the "Big 6", are regarded as the greatest concerns in this category (Elder et al., 2016). there

The STEC are distinguished from nonpathogenic *E. coli* strains by the production of Shiga toxin 1 (Stx 1), Shiga toxin 2 (Stx 2) or combinations of these toxins (Mellor et al., 2016;

Wang et al., 2002). Although no evidence has suggested that O antigens play a role in the virulence of pathogenic STEC, there are various factors and toxins contributing to their increasing virulence and pathogenicity (Mellor et al., 2016; Bosilevac et al., 2002). The STEC that produce Stx 2 alone or both Stx 1 and Stx 2 are more likely to be associated with HUS than those that produce Shiga toxin 1 (Stx1) alone (James et al., 2006). Other than presence of Shiga toxin, other key virulence factors include enterohemorrhagic *E. coli* hemolysin (EHEC *hlyA*) and intimin. As the expression product of the *eaeA* gene, intimin is involved in the attaching and effacing adherence to the host cell (Wang et al., 2002). According to Mead et al. (1998), a 60 MDa virulence plasmid (*pO157*) and the products of the pathogenicity island called the locus of enterocyte effacement (LEE), also contribute to the virulence of *E. coli* O157:H7 (Mead et al., 1998). The *Microbiology Laboratory Guidebook* (MLG) published by USDA-FSIS employs virulence gene sets of Shiga-toxin producing genes (*stx*), an attaching and effacing gene (*eae*), and genes specifically encoding each of the top 6 STEC serogroups and the O157 serogroup as a standard for detection of top 7 STEC (Rivas, 2015; USDA, 2019).

E. coli O157:H7

E. coli O157:H7 is so named based on its expression of the 157th somatic (O) antigen identified and the 7th flagellar (H) antigen. In 1982, *E. coli* O157:H7 was first recognized as a human pathogen (Mead et al., 1998). Subsequently, in 1983, Karmali et al. (1983) reported an association between infection with *E. coli* O157:H7 and HUS (Mead et al., 1998). Cattle were first described as a reservoir of *E. coli* O157:H7 STEC in 1987 (Borczyk et al., 1987). Later, in 1993, a devastating multi-state outbreak *E. coli* O157:H7 resulted in more than 700 illnesses and deaths of four children; the outbreak was linked to undercooked hamburger patties from a fast-food restaurant chain (Rangel et al., 2005). This outbreak made *E. coli* O157:H7 widely-

recognized as a life-threatening pathogen and challenged the government's approach to food safety, diagnosis of foodborne disease and outbreak monitoring. As a result, a series of reformed regulations and policies for better monitoring and control of pathogen contamination was published. After the 1992-1993 outbreak, *E. coli* O157:H7 was declared to be an adulterant in ground beef and raw non-intact beef products, and a zero tolerance policy was implemented by USDA-FSIS (Mellor et al., 2016; Wheeler et al., 2014). As systems were established for surveillance of adulterant *E. coli* O157:H7 in beef products, adulterant STEC O157 strains were characterized by the presence of *stx, eae*, and the O157 antigen marker (Mellor et al., 2016).

According to CDC (2020a), an estimated 265,000 STEC infections occur each year in the U.S.. The STEC O157 causes about 36% of these infections, and non-O157 STEC cause the rest. Other than consumption of undercooked beef, *E. coli* O157:H7 outbreaks have also been traced to non-meat sources, such as fruits and raw vegetables, unpasteurized milk and juice, and unchlorinated water (CDC, 2020b). Prevalence of *E. coli* O157:H7 has been reported to be susceptible by seasonal factors. The peak prevalence for this pathogen in feces occurs in the summer, whereas its prevalence on hide was high from the spring through the fall.

Non-O157 STEC

Non-O157 STEC can cause several foodborne illnesses that are comparable to *E. coli* O157:H7 infections (Brooks et al., 2005). As more foodborne outbreaks are linked non-O157 STEC, importance of pathogenic non-O157 STEC has been recognized (Stromberg et al., 2018). The top six STEC serogroups in the U.S. are responsible for approximately 75 % of total non-O157 STEC illnesses U.S. annually (Brooks et al., 2005). An investigation conducted by Luna-Gierke et al. (2014) revealed that, from 1990 through 2010, 46 outbreaks related to non-O157 STEC were responsible for 1727 illnesses and 144 hospitalizations. Among those outbreaks,

serogroups O111 and O26 accounted for the highest proportion (66%) of outbreaks, followed by O45, O103, O121, O145 (Luna-Gierke et al., 2014). Similar to *E. coli* O157:H7, prevalence of non-O157 STEC on beef carcass are also influenced by season, and the peak occurs in the fall (Barkocy-Gallagher et al., 2003). Among the various detection methods developed for determination of top 6 STEC, PCR targeting genes-encoding O-antigen of top 6 serogroups has proven to detect with very high sensitivity. Most PCR methods employ primers that target the *wzx* and wzy genes in the biosynthetic operons of O26, O45, O103, O121, and O145, and open reading frame of the *E. coli* O111 *rfb* region (Bosilevac and Koohmaraie, 2012).

Aerobic Count

The Aerobic Plate Count (APC), also referred as the standard plate count, aerobic colony count, mesophilic count or total plate count, is used as an indicator of bacterial populations on meat samples. Although not serving as a safety indicator in food products, APC are one of the most important indexes in providing information on the flaws in process control systems or deficiencies in sanitation systems (Bird et al., 2016). In other words, APC are a good indicator of meat spoilage (Wang et al., 2011). Because APC represent organisms that grow at mesophilic temperatures (30–45 °C) in an aerobic environment, it is regarded as a reliable indicator of the overall level of bacterial contamination in a meat sample (Magwedere et al., 2013). Although APC are used widely to access microbial loads on fresh meat and poultry products, they don't indicate and differentiate types of bacteria (Jay et al., 2002). However, APC are still important for determining handling and storage history, overall product quality and for providing possible information on product safety and shelf life (Jay et al., 2002).

For purposes of determining the APC, several enumeration methods have been developed and shown as comparable to each other. The most-probable-number method relying on positive

number of tubes from a serial dilutions of inoculated samples is a statistical enumeration method used to estimate the total aerobic count (Karunasagar et al., 2018). In Chain and Fung's study conducted in 1988, they compared four alternative methods (i.e., Redigel, Petrifilm, Spiral Plate System, and Isogrid) with the widely used Aerobic Plate Count (APC) method for enumeration of aerobic counts from several matrixes of food sources such as chicken, ground beef and ground pork (Chain et al., 1991). Their study showed that those five enumeration methods were comparable to each other ($r = 0.97$), except for the correlation coefficient ($r = 0.88$) for Petrifilm versus Spiral Plate System. Instead of measuring the entire bacterial population, the APC method is actually counting colonies for organisms that grow aerobically at mesophilic temperatures (20 to 45°C). According to U.S. Food & Drug Administration, the APC method using plating count agar is regarded as a standard method for determination of aerobic mesophilic populations, and the incubation condition is 48 ± 3 h at 35 ± 1°C.

TEMPO AC Method for detection of aerobic count

Other than enumeration methods mentioned above, a recently developed automated computer-operated system (TEMPO, bioMe´rieux, Marcy l'Etoile, France) has been reported as comparable to the traditional methods for enumeration of APC (Crowley et al., 2009; Katase et al., 2011). As one of the traditional enumeration methods, the most-probable-number method has been applied for several decades. However, it has the disadvantage being time-consuming, expensive, and laborious because inoculations are required for multiple series of tubes at different dilutions.

To save time and labor cost, the TEMPO system uses the automated most-probable-number determination for micro-organism enumeration (Line et al., 2011). Briefly, this system,

with the ability to allow rapid enumeration within 24 h, is comprised of the TEMPO Filler, TEMPO Reader, barcode reader, computers, software and accessories (Katase et al., 2011). The TEMPO test consists of a vial of dehydrated culture medium and a microchannel card unique to the specific test (Crowley et al., 2010). The TEMPO AC test is specifically used for enumeration of aerobic mesophilic populations. For example, the vial for the TEMPO AC test contains culture medium in which the substrate will be hydrolyzed once there is aerobic organisms present during incubation and therefore the generated fluorescent signal will be detectable (Crowley et al., 2009).

The TEMPO card is a miniature MPN tool containing 48 wells of three different volumes and there is one-log difference between each set of volumes. In a TEMPO AC test, the culture medium is rehydrated by sterile water and inoculated with the test sample, which is automatically introduced onto the cards by an automated vacuum chamber (i.e., TEMPO Filler) (Crowley et al., 2009; Line et al., 2011). After filling, the straws of TEMPO AC cards are cut off to avoid risk of contamination. The cards on a card holder should be incubated at 35 ± 1°C for 22-28 h. The TEMPO Reader normally takes ~15 min for detection of the produced fluorescent signal at the end of incubation. The TEMPO software calculates the number of total positive wells and automatically estimate the sample based on the MPN estimate method (Crowley et al., 2009; Line et al., 2011).

Katase and Tsumura(2011) compared the TEMPO MPN methods and standard plate method for enumeration of five types of micro-organisms (i.e., APC, total coliforms, *Enterobacteriaceae*, yeast and mold and *Staphylococcus aureus*) from artificially contaminated soy product samples and naturally contaminated processed soy products on the market. They

found that TEMPO methods were equivalent to the corresponding standard plate methods with very good rates of agreement.

Detection and Characterization of Foodborne Pathogens

Early detection of foodborne pathogen is crucial to avoiding foodborne illness and major economic impact. For the meat industry, a rapid and reliable testing method for detection foodborne pathogens is very necessary because distribution delays and economic impacts caused by holding products diagnosed as presumptive positive must be managed in a controlled food safety plan. Until now, testing methods for detection of foodborne pathogens have been developed and enlarged. Among them, culture-based methods are the oldest methods and represent the "gold standard" diagnosis when positive results are obtained (Laupland et al., 2013). However, this method can be time-consuming and high-cost on labor and money. More recently, novel testing methods such as enzyme-linked immunosorbent assays (ELISA) and molecular biology-based methods, including polymerase chain reaction (PCR), are increasingly being used. In some cases, these new techniques are powerful with potential advantages and even replacing traditional testing methods (Laupland et al., 2013). However, it should be noted that every method has their limitations and no method can reach 100% sensitivity and specificity in real world.

Traditional culture method

With high-success rates for identification and differentiation of bacteria**,** culture methods can be used alone for presumptive diagnosis or as further confirmation steps, accompanied by other testing technologies (Laupland et al., 2013). Culture of *E. Coli* O157:H7 on Sorbitol MacConkey agar (SMAC) is one of the best examples of a high-performing method, which relies

on the principle of fermentation of sorbitol to differentiate the sorbitol-negative serotype *E. coli* O157:H7. As a result, colorless colonies are seen with presence of *E. coli* O157:H7 which is differentiated from fecal *E. coli* strains that show pink color because of fermentation of sorbitol (March et al., 1986).

As more non-O157 STEC strains are linked to severe clinical illness and outbreaks (e.g., *E. coli* O104:H4 Outbreak in Germany in 2011), reliable and rapid testing method of non-O157 STEC isolates in food and clinical samples is required. Compared with SMAC, the chromogenic media in the CHROMagar STEC Agar contains antimicrobials inhibiting the growth of fecal coliforms and other bacteria and therefore, the majority of STEC strains are seen in mauve colony color, while in general other bacteria are colored in blue or inhibited (Hirvonen et al., 2012). Generally, the chromogenic medium, referred as CHROMagar, is offering easier discrimination based on color and higher specificity and sensitivity for STEC screening compared to traditional SMAC (Priyanka et al., 2016). Long incubation periods (at least 18 – 24 h) are normally required using the culture method, which may result in significant adverse effects on outcome of severe disease (Laupland et al., 2013). Other than that, culture methods alone may not be as sensitive as a PCR method, especially for detection of low concentrations of pathogenic organisms such as *E. coli* O157:H7 (Holland et al., 2000). However, the ability of strain isolation always makes the culture method as a consideration for further outbreak investigations (Hirvonen et al., 2012).

Immunoassay method

Immunoassay method is less expensive and faster than traditional culture method, which is normally applied before PCR screening (Priyanka et al., 2016). Enzyme linked immunosorbent

assay (ELISA) is one of the most widely used immunoassays in laboratories. In general, the ELISA reaction is a process in which the enzyme-labeled antibodies combine with target antigen, and the color change of substrate indicates the enzyme activity when positive sample is present. A study conducted by Park et al. (1996) compared performance of ELISA method with conventional SMAC culture method on detection of *E. coli* O157:H7. In this study, the ELISA method successfully reduced the false negative rate generated by SMAC and reached specificity and sensitivity of 99.5% and 91.2%, while the result for SMAC culture method was 100% and 82.4%, respectively.

Molecular PCR method

Polymerase chain reaction (PCR) has been considered a rapid and sensitive approach to detect and identify pathogens in food safety laboratories (Priyanka et al., 2016). A normal PCR process consists of three repeating steps: denaturation, annealing and elongation. Generally, the detection of bacterial pathogens by PCR is the process in which specific designated DNA primers and probes are used, along with substrate and DNA polymerase, in heating-cooling cycles to amplify the target DNA (Laupland et al., 2013; Brooks et al., 2020). Specific primers are used for targeting different genes, and therefore, it's important to have well-designed primers not amplifying non-target sequences. The primers used for probe amplification are designated based on virulence gene targets.

With high sensitivity and specificity, real-time qPCR is widely used for detection and quantification of microorganisms such like STEC (Priyanka et al., 2016). In real-time PCR, fluorescent labeling enables the collection of data as PCR progresses, because fluorescent signal increases in direct proportion to the amount of PCR product is amplified (Qin, 2006). Otherwise,

designated internal control is normally used for monitoring inhibition in each sample and indicating false positive or false negative results (Omar et al., 2014). A well-designed internal control can indicate the inhibitory factor in a sample that may cause false-negative results and therefore, additional retesting of the same sample could increase sensitivity of the PCR method (Rosenstraus et al., 1988).

Among many detection methods against top 7 STEC, PCR screening method is the fastest approach with high sensitivity. Many commercial kits have been developed and validated for further usage in meat industries and regulatory agencies. The detection of STEC in samples primarily relies on the following steps: enrichment, detection and culture confirmation (Bouvier et al., 2017). Many commercial kits detect *stx*, *eae,* and genes encoding O-antigen for determination of top 7 STEC following the MLG protocol published by FSIS (Wheeler et al., 2014). However, one disadvantage of current testing workflow is that false positives result can be generated due to presence of one or two target genes from independent cells (Livezey et al., 2015). False positives are generated when the PCR screening result does not match with the culture method, and in this scenario, non-confirmable samples can cause economic loss due to hold or total loss of beef products. To fix this issue, new assays targeting additional virulence genes associated with EHEC have been developed. For example, a study conducted by Delannoy et al. (2016) revealed that screening for *stx*, *eae*, *espK*, and *espV*, in association with the *CRISPRO26:H11* marker is a better testing approach to decrease the potential positives generated from PCR screening in beef enrichments. Once the screening step is narrowed down, there is a potential that false positive rate decreased accordingly.

CHAPTER 2: EVELUATION OF THE TEMPO® AC TEST FOR ENUMERATION OF TOTAL AEROBIC MESOPHILIC MICROBIAL POPULATION FROM MICROTALLY SHEETS USED TO SAMPLE BEEF CARCASSES AND HIDES

Summary

An automated most-probable-number (MPN) system TEMPO (bioMérieux, Marcy l'Etoile, France) has been developed for enumeration of bacterial populations in food products and environmental samples. In the current study, we evaluated the automated TEMPO® AC Test in comparison with traditional direct agar plating method for enumeration of aerobic mesophilic flora in MicroTally sheets used to sample beef carcasses and hides. A total of 160 MicroTally (MT) sheet samples were collected from commercial beef processing plants by swab-sampling on the surface of naturally contaminated pre-evisceration carcasses, hides and post-chill final carcasses, and analyzed within 24 h after sample collection. Of these, all 160 samples were within detection limit and analyzed by both automated TEMPO AC test and traditional direct agar plating method. For these results, the aerobic count correlation coefficient was high (0.93) for pre-evisceration carcasses, which had mean (± standard deviation) counts of 3.3 ± 0.9 and 3.1 ± 0.8 log CFU/mL for those two methods, respectively. The aerobic count correlation coefficients were higher (0.95 and 0.96) for MT samples from hides and post-chill final carcasses, which had mean (± standard deviation) counts of 5.3 ± 1.2 and 5.0 ± 1.2, 3.0 ± 1.4 and 3.0 ± 1.3 log CFU/mL for those two methods, respectively. Overall, 98.8% of aerobic count results were within 1-log difference between the two enumeration methods. The correlation coefficient (r = 0.97) and linearity regression (log TEMPO MPN/mL = 1.06 x log PCA-CFU/mL +0.03) for our whole sample set (n = 160) was calculated. Our results demonstrated that the automated MPN method-TEMPO AC Test generated total aerobic mesophilic microflora counts that were highly

correlated and consistent with counts obtained by traditional plating methods on enumerating total aerobic mesophilic microbial populations recovered from MicroTally sheets. The use of TEMPO AC test for MicroTally sheet analysis could provide the meat industry with more potential advantages in microbial analysis in terms of saving time and labor.

Introduction

Viable aerobic mesophilic flora are an important quality indicator in the food industry. Use of APC's to indicate hygiene conditions and status are common (Refai, 1979). Traditionally, enumeration of aerobic mesophilic flora can be achieved using several methods such as conventional standard plate incubation methods on selective medium, Petrifilm and MPN calculations (Park et al., 2001; Swanson et al., 2001; Tran et al., 1996). Conventional plating is a direct way for estimating microbial populations and has been applied for decades in various food samples. However, since conventional plating is labor-intensive and takes longer for sample preparation and analysis, it's necessary to develop effective methods as alternatives to try and reduce costs associated with testing in the food industry; particularly in test-and-hold programs. In contrast with manually-operated plating methods, automated enumeration requires less labor and time for enumeration of microorganisms.

An automated enumeration system TEMPO (bioMérieux, Marcy l'Etoile, France) has been developed based on a most-probable-number (MPN) determination. Paulsen et al. (2006) successfully used the TEMPO system to determine total aerobic bacteria (APC) on carcass surfaces and in minced meat. Previously, research has shown that the TEMPO MPN method can be an alternative method to traditional plating for the purpose of saving time and labor, and reducing costs of testing (Cirolini et al., 2010; Crowley et al., 2013). Each type of TEMPO test consists of a TEMPO card and a TEMPO vial containing selective culture medium. The TEMPO

card is a miniature MPN tool with 48 wells of three different volumes, and there is a 1-log difference between each volume. Each well of the TEMPO card is inoculated with the testing sample in an automated vacuuming chamber (Line et al., 2011). After incubation, TEMPO cards are placed in a reading instrument connected to a computer for calculating an estimated MPN automatically.

The automated inoculation and enumeration mechanism attains the TEMPO MPN method the advantages in saving time and labor work (Crowley et al., 2009; Owen et al., 2010). A study conducted by Line et al. (2011) found that when analyzing the same amount of samples, TEMPO requires less people to set up a test (1 trained person for TEMPO test versus 4 trained people for traditional methods), as well as less time for counting colonies manually.

In addition to food products, the TEMPO system is expected to be expanded to analyze specific microbial populations in various matrices such as in food sampling devices. In the meat industry, N60 is a sampling method currently utilized by companies that handle beef trimmings to collect and composite 60 or more surface excision slices from the exterior of trimmings, up to a total weight of ~375 g for pathogen testing (sampling method-dependent; Wheeler and Arthur, 2018). However, this excision sampling method (i.e., N60 or N60 Plus) causes concerns regarding product loss and workforce safety during sample collection (Wheeler and Arthur, 2018). Therefore, it is necessary to find an alternative method for sampling meat products. The MicroTally (MT) sampling device, a nondestructive spunbond olefin polymer cloth, has been developed as an alternative to the current standard N60 and N60 Plus method. In a study conducted by Wheeler and Arthur (2018), MicroTally sheets were applied to swab beef trimming samples and enumerated for aerobic plate count plated on Petrifilm. Their results showed that

MT sampling device is comparable or even better relative to microorganism recovery than the current standard N60 and N60 Plus method (Wheeler and Arthur, 2018).

The objective of the current study was to evaluate the automated TEMPO® AC Test (bioMerieux, Marcy-l'Étoile, France) for its enumeration of aerobic mesophilic flora in a new matrix, MicroTally sheets used to sample beef carcasses and hides, by comparing with traditional direct agar plating method.

Materials and Methods

Sample collection

A total of 160 samples were collected from two beef processing plants in Nebraska on four collection days using MicroTally (MT) sheets with the manual sampling device (MSD) method (Wheeler and Arthur, 2018). In the current study, the MT sheets were used to swab-sample the surface of carcasses at several beef harvesting process sampling sites, including pre-evisceration carcasses (4,000 cm^2), post-chill final carcasses (4,000 cm^2) or hides (2,000 cm^2). Following sampling, MT sheets were returned to their sterile plastic bags and were shipped to Colorado State University (CSU; Fort Collins, CO) for analysis.

Samples 1 to 72 were collected from pre-evisceration carcasses on two collection days (S1-S25 and S26-S72). Samples 73 to 122 (S73-S122) were collected from the hides of cattle before hide removal. Samples 123 to 160 were collected from chilled carcasses in the carcass holding/sales cooler on a single collection day. All MT samples in individual sample bags were shipped overnight, in a cooler with ice bricks or ice packs, from Nebraska to CSU for processing.

MT sample processing

A total of 160 MT samples were processed immediately after they were delivered to the Food Safety & Microbiology Laboratory of the Center for Meat Safety & Quality at CSU. A 200-mL volume of full-strength buffered peptone water (BPW; Difco, Becton Dickinson, and Company [BD], Sparks, MD) was added to each of the MT sample bags, followed by mechanical pummeling (Masticator, IUL Instruments, Barcelona, Spain) for 1 min. A 5-mL aliquot from each sample then was transferred into individual sterile tubes and appropriately diluted, in 0.1% BPW, before microbiological analysis with the automated most-probable-number (MPN) method using TEMPO and the traditional plating method.

TEMPO automated enumeration of total aerobic mesophilic microbial populations (MPN/mL)

The TEMPO® AC (Aerobic Count) test, conducted per the manufacturer's instructions, was used for enumeration of total aerobic mesophilic microflora. On each of the sample analysis days, TEMPO AC vials containing dehydrated culture medium and sterile deionized water were taken out of the refrigerator and were allowed to reach room temperature before use. A 3-mL volume of sterile water, as secondary diluent, was added into each TEMPO AC vial, using a dispenser, for rehydration of the AC culture medium. Then, 1-mL aliquots of undiluted or diluted MT samples were added to individual vials, using a sterile pipette, followed by mixing for 3 s on a vortex mixer. Sample information (i.e., sample ID and dilution level) were entered manually into the TEMPO system at the workstation.

Barcodes on the TEMPO AC vials and TEMPO cards were scanned into the system for each TEMPO AC test. In this manner, the TEMPO AC vials and TEMPO cards were associated with the corresponding samples. After scanning, the vials and corresponding cards were placed

into a customized rack with a capacity for six samples. The rack was then placed into the TEMPO Filler instrument for vacuuming. During the vacuuming process, each TEMPO AC card with 48 wells of three different volumes was automatically filled with the sample from the corresponding vial. After the TEMPO AC cards were filled, the cards were kept upright in incubation racks and incubated at 35°C for 22-28 h, per the manufacturer's instructions. After incubation, the racks with cards were inserted in the TEMPO Reader instrument for detection of the fluorescence signal.

Wells of cards with detectable fluorescence signal were interpreted as "positive" by the software, indicating presence of microbial growth. Depending on the number and type of the positive wells, TEMPO Read software provided an estimated result, in CFU/mL, for the undiluted or diluted MT samples. Total aerobic mesophilic microflora counts in original, undiluted MT samples were calculated by multiplying the TEMPO automated CFU/mL value by the dilution factor. It should be noted that, although the unit of output obtained with the TEMPO method was CFU/mL, for purposes of this study, we used MPN/mL to describe TEMPO results in order to differentiate these results from those obtained with the traditional plating method, which we report here as CFU/mL.

Traditional plating method for enumeration of total aerobic mesophilic microbial populations (CFU/mL).

Undiluted or diluted MT samples were spread-plated onto plate count agar (PCA; Difco, BD) plates and incubated at 35°C for 48 h. A 1-mL volume of the undiluted MT samples was spread-plated over three PCA plates, while a 100-µL aliquot of diluted samples was spread-plated, in duplicate, onto a PCA plate. PCA plates with between 25 and 250 colony-forming

units (CFU) were considered as acceptable for counting. However, data are still valid if the plates from the highest dilution level obtained over 250 CFU, or the plates from the lowest dilution level obtained lower than 25 CFU. Total aerobic mesophilic microflora counts in the original, undiluted MT samples, were calculated by multiplying the number of CFU/mL from plates by the dilution factor. When plates from consecutive dilutions had CFU within the countable range, the average of the calculated CFU/mL value from each dilution level was used for statistical analysis.

Statistical analysis

Data from each enumeration method was entered into an Excel (Microsoft, Redmond, WA) spreadsheet and log transformed. Since the TEMPO MPN method has an enumeration range of 1 to 4,900 MPN/mL (equivalent to 0-3.7 log MPN/mL), results of tests could be grouped as within, below or above the detection limit. When estimated MPN counts of original MT samples (undiluted) were <1 MPN/mL (equivalent to <0 log MPN/mL), the TEMPO result was considered as below detection limit. When estimated MPN counts for the highest dilution level of a sample were >4,900 MPN/mL (equivalent to >3.7 log MPN/mL), the TEMPO result was considered as above the detection limit. When the estimated MPN count fell between 1 to 4,900 MPN/mL, the TEMPO results were considered within the detection range. Additionally, counts also were considered as below detection limit if no colony growth (i.e., 0 CFU) was obtained for the lowest sample dilution plated on PCA plates.

For samples generating counts falling within the range of detection, criteria similar to those described by Paulsen et al. (2008) were used to define the potential correlation between the two methods. When the difference between the count obtained with the TEMPO MPN method

and traditional plating method exceeded 1 log CFU/mL, results were considered to be a "discrepancy"; when the difference was less than or equal to 1 log CFU/mL, results were considered to be in "agreement" (Paulsen et al. 2008). The R studio (version 1.2.5019) was used for statistical comparisons of results. Results for samples with counts that were within the detection range were log transformed. Least squares means, standard deviations, linear regression equations, and appropriate correlation coefficients (Pearson's product-moment correlation) were computed in R studio. Paired t-tests were performed on each sampling site using a significance level of $\alpha = 0.05$.

Results and Discussion

In this study, automated TEMPO AC was compared to direct agar plating for enumeration of total aerobic mesophilic microbial populations in MT sheets collected from beef carcass surfaces or hides. Overall, a total of 160 sheets were analyzed by both methods. As described above, we considered the differences between TEMPO and PCA plating counts i) >1 log-unit as discrepancy and ii) ≤ 1 log-unit as agreement (Paulsen et al. 2008). As shown in Table 2.1, discrepancy rates for pre-evisceration carcass, hides and post-chilling carcass samples were 1.4% (1 out of 72 samples), 2.0% (1 out of 50 samples) and 0% (0 out of 38 samples), respectively. A study conducted by Paulsen et al. (2006) implied that 5% discrepancies in results are considered as tolerable at the 1.0-log difference level based on the Campden guideline. Therefore, our results for each sampling site yielded discrepancies of < 5% which also implied acceptability at the 1.0-log difference level. A study conducted by Line et al. (2011) showed that the TEMPO MPN counts are consistent with traditional methods of enumeration when 97.5% and 93.7% of the *E. coli* and TVC (Total Viable Counts) results were < 1.0 log unit difference.

As a result, performance of TEMPO MPN in our study was comparable to previous research because 98.8% of our results were < 1.0 log unit difference.

Our overall correlation coefficient factors indicated that the two analytical methods correlated very well (r >0.93) for both samples with lower microbial loads from pre-evisceration and post-chill final carcasses (~3 log CFU/ml) and samples with higher microbial loads from hides (~5 log CUF/ml). The detailed correlations between automated TEMPO AC and direct plating counts for each MT sheet sample are shown in Figure 2.1 utilizing log-transformed data. Most observations (158 out of 160 samples) fell within the bracket indicating that the difference between automated TEMPO AC and direct agar plating counts were less than 1 log CFU/mL. As is shown in Figure 2.1, the slope for the regression model changes when PCA plating result of 5.0-log CFU/mL is used as cut-off criteria on each sampling site. Therefore, correlation coefficients were calculated separately for all samples with PCA plating counts above and under 5.0-log CFU/mL. As a result, for all 160 samples tested in this study, 131 samples obtained PCA plating result less than 5.0-log CFU/mL, while 29 samples resulted PCA plating result greater than 5.0-log CFU/mL. The correlation coefficients were 0.94 and 0.65 for samples with PCA plating result less and greater than 5-log CFU/mL, respectively, indicating that the correlation between TEMPO AC method and the traditional plating method was better on testing samples with lower microbial loads than those with higher microbial loads.

For MT samples collected from pre-evisceration carcasses (correlation coefficient r= 0.93), the mean counts (± standard deviation) obtained by TEMPO AC method and traditional plating method were 3.3 ± 0.9 and 3.1 ± 0.8 log CFU/mL, respectively (Table 2.2). For MT samples collected from hides (r= 0.95), mean counts (± standard deviation) were 5.3 ± 1.2 and

5.0 ± 1.2 log CFU/mL, respectively (Table 2.2), for each enumeration method. For MT samples collected from post-chill final carcasses (r= 0.96), mean counts (± standard deviation) were 3.0 ± 1.4 and 3.0 ± 1.3 log CFU/mL, respectively, for each enumeration method (Table 2.2). As expected, aerobic mesophilic microflora counts from hide samples (~5.3 ± 1.2) were greater (P <0.05) than those from pre-evisceration carcasses (~3.3 ± 0.9) and post-chilling carcasses (~3.0 ± 1.4) samples by TEMPO AC test (log MPN/mL). Although a previous study conducted by Line et al. (2011) showed that sample sets with higher mean counts (3.09 log MPN/ml) had a higher correlation factor of 0.972 between the two analytical methods, a correlation factor of 0.710 was obtained for those samples with lower mean counts (1.53 log MPN/ml). Such a trend was not obvious for the data obtained in our study.

Katase and Tsumura (2011) obtained results showing good rates of agreement for TEMPO TVC and standard plate count methods (r = 0.95, linear regression equation: log (TEMPO-TVC CFU g^{-1}) = 1.00 x log (standard count agar CFU g^{-1}) + 0.35) for enumeration of aerobic counts from naturally contaminated processed soy products. To enable comparisons with previous studies, we developed correlation coefficients (r = 0.97) and linear regression equations (log TEMPO MPN/mL = 1.06 x (log PCA-CFU/mL) +0.03) for our whole sample set (n= 160). The linear regression equation for each sampling site is shown in Figure 2.1.

Mean values from the TEMPO AC MPN method were greater than those from traditional method for MT samples collected from pre-evisceration carcasses and hides (Table 2.2). This observation could possibly be explained by the fact that some samples were more easily analyzed with specific systems (Chain et al., 1991). Herbret mentioned that the MPN method frequently generates greater count estimates than plate counting due to the fact the microorganisms will

continue to grow since they are not disrupted into individual cells in the growth medium of MPN method (Herbert et al., 1990). Moreover, no difference was detected for MT samples from post-chill final carcasses ($P > 0.05$) for the two enumeration methods.

Generally, TEMPO MPN have been successfully used in several matrixes of food samples such as meat, poultry, vegetables and dairy products to estimate *Enterobacteriaceae,* coliform counts or total aerobic counts (Park et al., 2001; Paulsen et al., 2006; Owen et al., 2010). Performance of TEMPO AC method in our study for enumeration of aerobic counts in comparison with a traditional plating method was in agreement with several previous TEMPO studies.

In addition to the types of food matrices tested before, our study showed that TEMPO® AC Test can be used to enumerate mesophilic microflora counts in a new matrix of MT sheets. Recent study conducted by Wheeler & Authur (2018) showed that the MT sampling device could work equivalently or even more efficiently than the current standard sampling method (i.e., N60 and N60 Plus) on recovery of organisms from beef trimmings. Our results demonstrated that TEMPO® AC was consistent with traditional plating for estimating recoverable aerobic counts from MT sheet samples collected from pre-evisceration carcasses and hides with good rate of agreement. No difference was found in post-chill final carcass samples. In other words, TEMPO ® AC was equivalent to the traditional plating method on analysis of MT samples collected from post-chill final carcass samples. Considering results from these studies collectively, it suggested that TEMPO® AC Test, together with MT sheets, could provide the meat industry new options regarding sample collection and analysis with potential better performance than the current methods being used.

Conclusion

Our results demonstrated that the automated MPN method-TEMPO AC generated total aerobic mesophilic microflora counts that were highly correlated and consistent with the counts obtained by traditional plating methods that enumerate total aerobic mesophilic microbial populations from MicroTally sheets. Use of TEMPO AC test for MicroTally sheet analysis could provide the meat industry with potential advantages in microbial analysis relative to saving time and labor, and thereby reducing costs of testing.

Table 2.1: Agreement of results obtained from the automated MPN method (TEMPO) and traditional plating method (PCA plating) for enumeration of total aerobic mesophilic microbial populations from MicroTally sheets used to sample beef carcasses and hides.

Sampling sites	n	Agreement[a]		Discrepancy[b]	
		n	%	n	%
Pre-evisceration carcasses	72	71	98.6	1	1.4
Hides	50	49	98	1	2
Post-chill post-chill final carcasses	38	38	100	0	0
Total	160	158	98.8	2	1.2

[a] Agreement (≤1 log-unit difference between TEMPO and PCA plating counts)
[b] Discrepancy (>1 log-unit difference between TEMPO and PCA plating counts)

Table 2.2: Comparison of automated MPN method (TEMPO) and traditional plating method (PCA plating) for enumeration of total aerobic mesophilic microbial populations from MicroTally sheets used to sample beef carcasses and hides.

Measurement	Pre-evisceration Carcass		Hides		Post-chill final carcasses	
	TEMPO MPN[a]	Traditional plating[b]	TEMPO MPN[a]	Traditional plating[b]	TEMPO MPN[a]	Traditional plating[b]
Mean	3.3	3.1	5.3	5	3	3
Minimum	0.6	0.6	3.5	3.2	0.5	1
Maximum	5.3	4.9	7.1	6.2	5.5	5.5
SD	0.9	0.8	1.2	1.2	1.4	1.3
n	72	72	50	50	38	38
Correlation Coefficient[c]	0.93		0.95		0.96	

[a] TEMPO MPN: TEMPO MPN method (log MPN/mL)

[b] Traditional plating: Traditional plating method (log CFU/mL)

[c] Correlation Coefficient: Pearson's product-moment correlation between TEMPO MPN and traditional plating method

CHAPTER 3: EVALUATION OF GENE-UP SYSTEM FOR DETECTION OF SHIGA-TOXIN PRODUCING ESCHERICHIA COLI IN MICROTALLY SHEETS COLLECTED FROM BEEF CARCASSES

Summary

This study was conducted to determine specificity of bioMérieux's GENE-UP, a PCR-based molecular diagnostic system, to detect Shiga Toxin-producing *Escherichia coli* (STEC) from samples collected from beef processing plants using MicroTally sheets with the manual sampling device method. A total of 194 MicroTally (MT) samples were collected from beef processing plants and analyzed for determination of the top 6 STEC and *E. coli* O157: H7 (top 7 STEC) by GENE-UP system, BioRad and BioControl GDS commercial kits. Fifty MT samples were collected from swabbing pre-evisceration carcasses and inoculated with hide-derived inocula, while the remaining 144 MT samples were derived from post-chilling carcasses in sales coolers and inoculated with *E. coli* strains. All inoculated MT samples were enriched for 8-hr and 10-hr at 42°C in buffered peptone water (BPW) broth and re-collected after incubation. Eight-hour and 10-hr enrichment samples were analyzed using the GENE-UP system at Colorado State University and sent to U.S. Meat Animal Research Center (USMARC, Clay Center, NE) for detection of top 6 STEC and *E. coli* O157: H7. The GENE-UP system uses EH1 assay to detect *stx* and *eae* genes, ECO assay to detect genes specific to O157:H7 serogroup, and EH2 assay to differentiate top 6 serogroups. The NM-EHEC assay targeting virulence genes *espK, espV* and *CRISPR_O26E* does not directly differentiate top 7 STEC, but serves as additional screening test to help identify any organisms in a sample that are categorized as a "top 7 STEC." All potential positive samples determined by PCR screening were plated onto selective agar for culture

confirmation. After immunoconcentration step, isolates picked from selective agar were subjected to additional PCR screening. BioRad and BioControl GDS PCR screening methods were performed following their standard protocols for determination of top 7 STEC at USMARC. Presumptive positive samples confirmed by the additional PCR test were designated as "true positives." Presumptive positive samples that were not confirmed by the additional PCR test were designated as "regulatory false positives." Overall, our results indicated that the GENE-UP system worked well on determination of top 6 STEC and *E. coli* O157:H7 recovered from the MicroTally sheets. In order to reduce or eliminate false negative results, a 10 h enrichment time in BPW was required for detection of both the top 6 STEC and *E. coli* O157:H7. Compared to GENE-UP and GDS, BioRad generated a much higher number of potential positives that required cultural confirmation. In addition, use of the NM-EHEC kit targeting virulence genes (*espK, espV* and *CRISPR_O26E*), as an additional PCR screening after EH1 PCR (*stx* and *eae*), showed potential to reduce the number of samples that require further O-type determination. However, an adjustment that could improve GENE-UP *E. coli* O157:H7 detection would include reducing numbers of false negative results caused by the shift of T_m when *E. coli* O157:H7 and O157: non-H7 co-exist in a sample.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) infections cause clinical symptoms ranging from mild diarrhea to hemorrhagic colitis and, in serious cases, hemolytic uremic syndrome (HUS) (Tarr et al., 2005). Onset of HUS is a thrombotic disorder and is the most common cause of acute renal failure in children (Scheiring et al., 2008). Among STEC's, serotype *E. coli* O157:H7 is considered to be associated with HUS and is an important foodborne pathogen linked to many outbreaks in the U. S. (Tarr et al., 2005). In 1993, an *E. coli* O157:H7

outbreak was linked to undercooked ground beef and resulted in the deaths of four children (Rangel et al., 2005). Since then, *E. coli* O157:H7 was widely recognized as a life-threatening pathogen and zero tolerance policy was implemented by USDA-FSIS (Wheeler et al., 2014).

According to the CDC, STEC O157 causes about 36% of estimated 265,000 STEC infections each year in the U.S., while non-O157 STEC cause the remainder of STEC infections (CDC, 2020a). The top six STEC serogroups, including O26, O45, O103, O111, O121, and O145 (referred to as "Top 6" or "Big 6") are responsible for 75-80 % of total non-O157 STEC illnesses in the U.S. annually (Brooks et al., 2005). In 2011, the top six STEC serogroups (i.e., O26, O45, O103, O111, O121, and O145), were added to the list of adulterants on raw, non-intact beef product or components (Wheeler et al., 2014). Because presence of *E. coli* O157:H7 and top 6 non-O157 STEC (referred as top 7 STEC) in contaminated beef and other food source can cause severe disease, a rapid, accurate and sensitive detection approach on those pathogens is necessary for meat industries and regulatory agencies to ensure safe supply of foods (Boer et al., 2000).

Use of the polymerase chain reaction (PCR) is considered a rapid approach for detection of pathogenic STEC, and most commercial PCR kits focus on detecting virulence factor genes (Rivas, 2015). For example, the *Microbiology Laboratory Guidebook* (MLG) utilized by USDA-FSIS, employs primers targeting on Shiga-toxin producing genes (*stx*), an attaching and effacing gene (*eae*), and genes specifically encoding each of the top 6 STEC serogroups and the O157 serogroup (Rivas, 2015). Detection of Shiga toxin 1 (stx_1), Shiga toxin 2 (stx_2), and intimin (*eae*) helps to identify STEC because those genes are linked to pathogenicity of STEC strains (Beutin et al., 2004). Bosilevac et al. (2019) discussed that an enrichment sample identified as possessing *stx, eae* and one of six O serogroups or O157:H7 serogroup is considered to be a potential

positive detection of one of the top 7 STEC. Potential positive samples are then subjected to selective agar for cultural confirmation. Until the time that a colony from selective agar is confirmed using an additional PCR test, the sample is called presumptive positive. Only if the additional PCR test confirms the presumptive positive result from an isolated colony will the sample will be considered to be positive (Bosilevac et al., 2019).

A number of PCR-based detection methods have been developed to differentiate among the different Top-7 STEC's; however, no method is perfect and there is room for improvement of current detection methods (Peiyanka et al., 2016). For example, some molecular methods require cumbersome preparation of reaction mixture, while some employ tedious DNA extraction procedures to remove PCR-inhibiting compounds, or some may not include an internal amplification control (Fratamico et al., 2017). Bosilevac et al. (2019) revealed that many PCR-based assays relying on detection of genes involved in O157 surface antigen can generate false positive result because mixture culture of *E. coli* O157 and non-O157:H7 can cause false determination and that is not uncommon in beef product enrichment. Therefore, a more rapid and sensitive PCR approach would be valuable to the meat industries and regulatory agencies for detection of important pathogenic STEC.

The GENE-UP system evaluated in this study is based on real-time multiplex PCR and consists of three assays: EH1 assay using GENE-UP STEC *stx/eae* kit detects *stx* and *eae* genes, ECO assay using GENE-UP *E. coli* O157:H7 kit detects genes specific to O157:H7 serogroup, and EH2 assay using GENE-UP STEC-Top 6 kit detects and differentiates genes encoding O antigen of top 6 serogroups. The NM-EHEC assay using GENE-UP New Markers EHEC kit and targeting virulence genes *espK, espV* and *CRISPR_O26E* does not directly determine top 7 STEC, but serves as additional screening test to help identify top 7 STEC.

In this study, we introduced MicroTally sheets as the sampling device for recovery of top 7 STEC and performed the GENE-UP PCR screening system on this new sample matrix. The MicroTally device is a spunbond olefin polymer cloth, which has been shown to perform comparably, or even better than, the traditional N60 excision sampling method used in beef plants for recovery of pathogens (Wheeler & Arthur, 2018). The aim of this study was to determine specificity of bioMérieux's GENE-UP, a PCR-based molecular diagnostic system, to detect Shiga Toxin-producing *Escherichia coli* (STEC) from samples collected from beef processing plants using MicroTally sheets using the manual sampling device method.

Materials and Methods

Sample collection (conducted by USMARC)

A total of 194 samples were collected from two beef processing plants located in the central U.S. using MicroTally (MT) sheets and the manual sampling device (MSD) method (Wheeler & Arthur, 2018). The MicroTally sampling device is a cloth sheet composed of spunbond olefin polymer cloth (MicroTally swab; Fremonta Corporation, Fremont, CA). Advantages of this new sampling device are that it is nondestructive and it could be more efficient than the current N60 and N60 Plus sampling methods (Wheeler & Arthur, 2018).

Samples 1 to 50 (S1-S50) were collected from pre-evisceration carcasses after the pre-evisceration washing/rinsing by using unfolded MT sheets to swab an area of 2,000 cm^2. Samples 51 to 194 (S51-S194) were collected from the post-chilling beef carcasses in the sales/holding cooler using unfolded MT sheets to swab an area of 4,000 cm^2. The MT samples were blinded to protect their identities if necessary. The S1-S50 (samples 1 to 50) were inoculated with hide-derived inocula right after MT sample collection, and S51-S194 were sent

directly to Colorado State University (CSU, Fort Collins, CO) for inoculation with *E. coli* strains. All MT sheets (either inoculated or not) in individual samples bags were shipped overnight, in a cooler with ice bricks or ice packs, from Nebraska to CSU.

Inoculation of S1-S50 with hide-derived inocula (conducted by USMARC)

Hide-derived inocula were collected from hides of beef carcasses before hide washing. Ten pre-moistened sponges were used to swab a 500 cm^2 area of 10 separate hides. After swabbing of the hides, each sponge was manually massaged and an aliquot of the liquid portion of each sponge sample was used for inoculation of a subset of MT samples collected from pre-evisceration carcasses. Ten out of the 50 (S1-S50) collected MT samples were selected randomly for inoculation, and each received only the inoculum from one sponge sample. Two of the 10 MT samples were inoculated with 25 µL, each, and two with 50 µL, each, of hide inoculum. The remaining six MT samples were inoculated with 75, 100, 125, 150, 175, and 200 µL of the inoculum. All 50 MicroTally sheets (10 inoculated, 40 uninoculated) were then shipped overnight, in a cooler with ice bricks or ice packs, from Nebraska to CSU.

Inoculation of S51-S194 with E. coli Strains

A panel of *E. coli* strains, selected based on their serogroup, were used for inoculation of collected samples MT S51-S194 (from final beef carcasses). The panel of *E. coli* strains consisted of 29 unique strains from beef or cattle sources (Table 3.1). The MT samples obtained from post-chilling beef carcasses were collected on two collection days (designated as "Round 1" and "Round 2" in Table 3.1). The MT samples S51-S115 were collected in "Round 1", and MT samples S116-S194 were collected in "Round 2." Different sets of inocula were used to

inoculate MT samples S51-S115 and S116-S194, as shown in Table 3.1. Preparation of inocula consisted of serial dilution of overnight cultures of each *E. coli* strain to final concentrations of 100, 10, 1, 0.1, 0.01, and 0.001 CFU per vial. For each collection round, a total of 70 vials of inocula were prepared, containing different *E. coli* strains (Table 3.1) and concentrations. Vials were arbitrarily numbered and shipped to CSU to be added to each post-chill final carcass MT sample. A 370 µL volume of diluted *E. coli* inoculum, from individual vials, was added to individual MT sheet samples prior to the addition of the enrichment broth.

Sample Enrichment

A 200 mL volume of buffered peptone water (BPW; Difco, Becton Dickinson, and Company, Sparks, MD) was added to each MT sample bag inoculated either by USMARC or at CSU, followed by mechanical pummeling (Masticator; IUL Instruments, Barcelona, Spain) for 1 min. All samples were incubated at 42°C for 8 h and 10 h. After 8 h and 10 h of incubation, enrichment broth was collected from each sample and was aliquoted into several tubes for subsequent analyses or storage purposes (Table 3.2).

DNA extraction

DNA was extracted from 800 µL of the 8 h and 10 h BPW enrichment broths (hereafter referred to as 8 h and 10 h samples) using the automated VIDAS ESP1 assay (VIDAS®; bioMérieux, Marcy L'Etoile, France). The ESP1 assay is an automated immunoconcentration process capturing the top 7 serogroups (the top 6 STEC and *E. coli* O157) with utilization of the specific antibodies. The ESP1 assay serves as an immunoconcentraion and cell lysis process. When the ESP1 assay is complete, only DNA from the top 7 serogroups are released from the damaged cells, whereas other bacteria are washed out due to lack of top 7 O-antigen surface.

PCR screening for E. coli O157:H7 *and top 6 STEC using the GENE-UP system*

The GENE-UP system for detection of *E. coli* O157:H7 and the top 6 non-O157 STEC consists of three assays (i.e., EH1, ECO and EH2) that includes primers and probes for the stx_1 / stx_2, *eae*, and *E. coli* O157:H7 gene targets and top 6 O-antigen gene targets. For each assay, specific primers and dual fluorescence resonance energy transfer (FRET) hybridization probes were employed for the target sequences and internal control.

Immediately following DNA extraction, all samples were subjected to PCR analysis using the GENE-UP test kits, following the manufacturer's instructions. The EH1 assay, using the GENE-UP STEC *stx/eae* kit, was performed on all 8 h and 10 h samples for detection of *stx* and *eae* genes. Subsequent PCR assays were only performed on 10 h samples that were positive from the EH1 assay. For S1-S50, all EH1-positive samples were tested by ECO and EH2 assays, and the NM-EHEC assay. For S51-S194 (workflow in Figure 3.1), samples were first tested for the presence of *E. coli* O157:H7 using the ECO assay (GENE-UP *E. coli* O157:H7 kit). Negative samples from the ECO assay were then tested for presence of non-O157 top 6 serogroups using the EH2 assay (GENE-UP STEC-Top 6 kit). Testing was suspended on samples when both ECO and the following EH2 assays had a negative result. In addition, all EH1 assay-positive samples also were subjected to the NM-EHEC assay targeting virulence genes, *espK, espV* and *CRISPR_O26E* using the GENE-UP New Markers EHEC kit. Samples were defined as potential positives if they were positive for both EH1/NM-EHEC and ECO/EH2 by the end of PCR tests. For each PCR run, an *E. coli* O157:H7 strain Sakai (ATCC BAA-460) served as a positive control, and non-STEC *E. coli* K12 strain MG1655 (ATCC 47076) as a negative control for the EH1, ECO

and NM-EHEC assays. The *E. coli* O157:H7 strain Sakai was used as a negative control for the EH2 assay.

For purposes of presenting and describing results from testing of samples using the above combinations of individual assays, the following designations were assigned for the different testing scenarios:

- "GENE-UP-8 h", for samples enriched for 8 h and tested using the EH1 assay. Subsequent ECO and EH2 assays were performed on samples enriched for 10 h.
- "GENE-UP-10 h", for samples enriched for 10 h and tested using the EH1, ECO and EH2 assays.
- "NM-EHEC-10 h", for samples enriched for 10 h and tested using the EH1, NM-EHEC, ECO and EH2 assays.

Culture confirmation of samples positive for the EH1 assay (conducted by CSU)

Following PCR detection, all samples positive for the EH1 assay (*stx* and *eae* genes) were subjected to culture confirmation for *E. coli* O157:H7 or the Top 6 STEC using culture methods regardless of the presence of O antigen. For each sample requiring confirmation, 30 µL of viable cells immunoconcentrated by the VIDAS ESP2 assay from the enrichment broth were streak-plated on selective agars, namely plates of ChromID™ Coli and EZ-CHROM STEC Agar (Microbiology International, Frederick, MD). VIDAS ESP2 assay also relies on antibodies to capture the top 7 STEC. Instead of cell lysis, ESP2 assay release live cells after immunoconcentration. ChromID™ Coli and EZ-CHROM STEC plates were then incubated for 22-24 h at 35°C. Four to eight colonies were selected from either the ChromID™ Coli or EZ-CHROM STEC plates, following the manufacturer's instructions, and were purified for PCR verification. Presumptive positive samples confirmed by the additional PCR test were designated

as “true positives.” Presumptive positive samples that were not confirmed by the additional PCR test were designated as “regulatory false positives.”

PCR detection using BioControl GDS and Bio-Rad screening and culture confirmation (conducted by USMARC)

All samples were sent to the USMARC to be tested with one of two alternate STEC screening platforms. The 8 h samples were tested using BioControl GDS (GDS MPX Top 7 STEC and MPX ID assays), and the 10 h samples were tested using the BioRad IQ-check^{TM} PCR VirX and SerO STEC assays. Briefly, GDS MPX Top 7 STEC assay was targeting *eae, stx1,* and *stx2* and genes specific for *E. coli* O157:H7 after a IMS (immunomagnetic separation) -based preparation process that excluded organisms not belonging to top 7 serogroups. The GDS MPX Top 7 STEC assay differentiated *E. coli* O157:H7 from other top 6 serogroups, but not indicated specific O serogroup for top 6 STEC. Based on positive results from the GDS MPX Top 7 STEC assay, MPX ID assay used a mix of multiple markers to differentiate specific O serogroup after imunnoconcentration of top 6 serogroups (Feldsine et al., 2016). In general, the BioRad iQ-Check STEC VirX Kit detects virulence genes *stx1, stx2,* and *eae* after extraction of DNA, while the STEC SerO Kit is designated for differentiating six major STEC serogroups and *E. coli* O157. In addition to PCR screening, GDS beads with live cells for those potential positives were plated onto Rainbow agar and ChromSTEC agar plates, then colonies from those selective plates were selected and purified for PCR verification.

For purposes of presenting and describing results of testing, the following designations were assigned to the two alternate STEC screening platforms:

- "GDS-8 h", for BioControl GDS screening of the 8 h BPW broth for those EH1-positives by GENE-UP-10 h.
- "BioRad-10 h" for Bio-Rad screening of the 10 h BPW broth for all samples from S1-S115, and EH1-positives by GENE-UP-10 h from S116-S194.

Data analysis

For the 8 h and 10 h enriched samples, PCR results from GENE-UP-8 h, GENE-UP-10 h, NM-EHEC-10 h, GDS-8 h, and BioRad-10 h were compared with the results from culture confirmation as a reference. Performance determination was based on the following parameters (Bosilevac et al., 2019):

- sensitivity = 100 × (no. of true positives (TP) / [no. of TP + no. of false negatives (FN)])
- specificity = 100 × (no. of true negatives (TN) / [no. of TN + no. of false positives (FP)])
- FP rate = 100 × (no. of FP / no. of FP + no. of TN)
- FN rate = 100 × (no. of FN / no. of FN + no. of TP)
- positive predictive value (PPV) = 100 × (no. of TP / [no. of TP + no. of FP])
- negative predictive value (NPV) = 100 × (no. of TN / [no. of TN + no. of FN])
- test accuracy = 100 × (no. of (TP + TN) / total no. of sample tested)

To calculate confidence intervals for the method performance parameters such as sensitivity, specificity, and overall accuracy, the Wilson score with continuity correction was used (Bosilevac et al., 2019). Online tools, available at http://vassarstats.net/clin1.html, were used for these calculations.

Results and Discussion

Results of detection of Top 6 STEC in S1-S50 (Tables 3.3, 3.4 and 3.5)

The GENE-UP-10 h, BioRad-10 h and GDS-8 h platforms generated 13, 19 and 12 potential positives, respectively, for detection of the top 6 STEC in S1-S50 (Table 3.3). BioRad generated more potential positives requiring further cultural confirmations than the other PCR screening platforms. Although GENE-UP-10 h generated one more false positive compared with GDS-8 h, performance parameters such as sensitivity and false negative rate for these two platforms were the same (Table 3.5). With regards to sensitivity, GENE-UP-10 h (85.7%) seemed to perform slightly better than the NM-EHEC-10 h (78.6%) for detection of the Top 6 STEC (Table 3.5), when NM-EHEC-10 h generated one more false negative (Table 3.4).

Results of detection of E. coli O157:H7 *in S1-50 (Tables 3.3, 3.6 and 3.7)*

The GENE-UP-10 h, BioRad-10 h and GDS-8 h platforms generated 1, 10 and 10 potential positives, respectively, for the detection of *E. coli* O157:H7 in S1-S50 (Table 3.3). Among the 9 positive results confirmed by cultural methods, GENE-UP-10 h, BioRad-10 h and GDS-8 h generated 8, 1 and 1 false negatives, respectively (Table 3.6). Inoculation of hide-derived inoculum containing complex microbial communities could be one of the factors responsible for those false negatives generated. Eight false negatives were obtained by GENE-UP-10 h which was probably caused by the shift of T_m when a sample contained both *E. coli* O157:H7 and O157: non-H7 cells (Figure 3.2). As a result, false negatives obtained by GENE-UP-10 h caused a lower overall accuracy compared with BioRad-10 h and GDS-8 h (Table 7) on detection of *E. coli* O157:H7.

Results of detection of Top 6 STEC in S51-S194 (Tables 3, 8 and 9)

The GENE-UP-10 h, BioRad-10 h and GDS-8 h platforms generated 34, 62 and 31 potential positives, respectively, for detection of the top 6 STEC in S51-S194 (Table 3.3). BioRad had more potential positives requiring further culture confirmations than GENE-UP and GDS. As shown in Table 3.1, some of the inocula contained a mixture of two *E. coli* strains: one *stx*-positive but *eae*-negative, and the other *eae*-positive but *stx*-negative. Therefore, samples inoculated with these inocula would be expected to generate an enrichment broth that would allow detection of both the *stx* and *eae* genes; however, these samples would not be confirmed using cultural methods. In other words, specificity for each platform would not attain 100% in theory, because of these artificially introduced “false positives”.

The GENE-UP-10 h performed better than GENE-UP-8 h relative to detection of the top 6 STEC, especially considering the lower number of false negatives that were detected (Table 3.8). The GENE-UP-8 h generated a higher number of false negatives which adversely affected sensitivity and NPV (negative predictive value) compared with GENE-UP-10 h (Table 3.9). It was notable that, in addition to reducing false negatives, GENE-UP-10 h could also potentially generate more false positives, and therefore decrease specificity and PPV (positive predictive value).

No differences in performance parameters, including sensitivity, specificity, and the overall accuracy, were observed between NM-EHEC-10 h and GENE-UP-10 h (Table 3.9). These two platforms performed equivalently for detection of the top 6 STEC in S51-S194. However, of the 45 positives detected by GENE-UP EH1 for *stx* and *eae* genes, only 40 were also positive from NM-EHEC screening (data not shown). Fewer positives detected with the

NM-EHEC kit could help reduce the number of samples that require further serogroup determination, and therefore save operator costs and time.

These data show that, under the experimental conditions of the study, all performance parameters for GENE-UP-10 h seemed to be better than those of the BioRad-8 h and GDS-10 h platforms, offering improved sensitivity and overall accuracy when evaluating the top 6 STEC PCR screening against culture isolation results (Table 3.9). However, it was notable that enrichment conditions used in our study have been validated for GENE-UP, but not for BioRad and GDS. The MT is a new type of sampling device, and BPW enrichment of MT may not be the ideal enrichment conditions for BioRad and GDS.

Results of detection of E. coli O157:H7 *in S51-S194 (Tables 3, 10 and 11)*

The GENE-UP-10 h, BioRad-10 h and GDS-8 h platforms generated 10, 42 and 9 potential positives, respectively, for detection of *E. coli* O157:H7 in S51-S194 (Table 3.3). BioRad generated more potential positives requiring further cultural confirmation than GENE-UP and GDS.

When colonies were picked for culture confirmation, isolates positive for *stx* and *eae* were first tested for *E. coli* O157:H7, and then subjected to top 6 STEC determination. Samples confirmed as *E. coli* O157:H7 were not further tested for Top 6 STEC because each sample only received one inoculum type; either *E. coli* O157 or the Top 6 STEC (Table 3.1). Therefore, a total of 53 samples were subjected to O157 cultural confirmation and 42 non-O157 samples were subjected to the top 6 STEC cultural confirmation. As for result on *E. coli* O157:H7 detection, GENE-UP-10 h performed well in light of the fact that it produced reduced false negatives than GENE-UP-8 h, which was consistent with performance on top 6 STEC detection (Table 3.10).

Overall, based on PCR analysis of the 10 h enrichment samples, GENE-UP-10 h for detection of *E. coli* O157:H7 seemed to perform equivalently to the NM-EHEC-10 h (Table 3.11). Performance parameters, including sensitivity, specificity, and overall accuracy for GENE-UP-10 h were found to be better than those obtained for GDS-8 h (Table 3.11). The GENE-UP-10 h performed better than BioRad-10 h based on most of the performance parameters, including specificity and overall accuracy (Table 3.11). Although BioRad-10 h generated the highest (100%) sensitivity across all platforms, it was notable that BioRad-10 h generated 42 potential positives requiring further culture confirmation, while GENE-UP-10 h only generated 10 potential positives.

Discussion of detection on E. coli O157:H7 *and top 6 STEC in S1-S194*

The GENE-UP system targets the same genes as the FSIS MLG primer set and two other commercial test methods for detection of top 6 STEC and *E. coli* O157:H7 (Bosilevac et al., 2012). False negative results were observed by GENE-UP system, specifically for the ECO assay, in detection of *E. coli* O157:H7 from the S1-S50 that were inoculated from natural hide-derived inocula. The shift of T_m due to interference between *E. coli* O157:H7 and non- H7 STEC could be responsible for the false negatives generated. However, a study conducted by Bosilevac et al. (2019) performed the ECO assay in their study that successfully differentiated *E. coli* O157:H7 and O157: non-H7 when testing isolates samples (Bosilevac et al., 2019). Their data revealed that the ECO assay has the ability to discriminate *E. coli* O157:H7 from O157: non- H7 groups through an automated PMP analysis based on T_m data obtained from 3,113 isolates (Bosilevac et al., 2019).Their study also proved that the ECO assay performed comparable to the MLG reference method and offered improved sensitivity on detection of *E. coli* O157:H7 from enriched natural beef samples. Based on our result, since false negative results were generated

with co-existence of *E. coli* O157:H7 and non- H7 could, there is space for improvement of the ECO assay when it is applied to the mixed culture in "real-world".

It's not uncommon that mixed cultures exist in beef product enrichments, and as a result, the samples co-contaminated with two or more independent microorganisms that each contains only one or two target genes will cause a challenge to the current PCR screening approaches (Livezey et al., 2015). Today, many commercial PCR kits aim to detect gene sets published in the FSIS MLG protocol (i.e., *stx, eae,* genes specific for *E. coli* O157:H7 or genes encoding one of the top 6 O-antigen) for determination of *E. coli* O157:H7 and top 6 adulterant STEC. However, false positive results could be generated due to co-existence of target genes from independent microorganisms (Mcmahon et al., 2017). For example, false positives can be generated when there is *eae*-negative STEC and *eae*-positive *E. coli* in the same food sample.

To reduce false-positive rate on detection of adulterant top 7 STEC, many novel testing methods have been developed and validated for further use in the meat industry and by regulatory agencies. For instance, searching for additional target virulence genes associated with typical EHEC can effectively reduce false-positive rate (Mcmahon et al., 2017). The NM-EHEC kit evaluated in our study is a good example on fixing this issue. The NM-EHEC kit targets virulence genes (*espK, espV* and *CRISPR_O26E*), as an additional PCR screening after EH1 PCR (*stx* and *eae*), successfully decreased the potential positive samples requiring further O serogroup determination and therefore increased accuracy of PCR screening result. Otherwise, application of immunoconcentration process also aids in reducing false-positive issue. As was shown by our results, the BioRad platform normally generated more potential positives than GENE-UP or BioControl GDS, and that could be explained by a lack of immunoconcentration. The GENE-UP system utilized ESP1 immunoconcentration assay to target top 7 O-antigen,

while the GDS applied IMS to exclude those non-top 7 STEC, and, therefore, these two platforms generated fewer potential positives related to lower false positive rate.

Many researchers have focused on application of PCR testing method screening on top 7 adulterant STEC recovered from the beef product enrichment samples (Fratamico et al., 2017; Bosilevac et al., 2019). Our study showed that, in addition to the food matrix tested before, the GENE-UP real-time PCR system can be used to detect top 7 adulterant STEC from MicroTally sheets that were used to swab hides and beef carcasses. Overall, the aim of recovery of STEC from MicroTally sheets was achieved and our study suggested that the GENE-UP system, together with the MicroTally sheets, could provide the meat industry and regulatory agencies with new options for sample collection and top 7 STEC detection with potentially better performance.

Table 3.1: Escherichia coli isolates used for inoculation of MicroTally sheet samples S51-S115 (Round 1) and S116-S194 (Round 2)

Inocula	**Round 1**			**Round 2**		
	O group	*stx*	*eae*	O group	*stx*	*eae*
1	O74	+	+	O157:H7	+	+
2	O157:H7	+	+	O118	+	+
3	O157:H7	+	+	O111	+	+
4	O111	+	+	O26	+	+
5	O26	+	+	O157:H7	+	+
6	O103	+	+	O45	+	+
7	O45	+	+	O157:H7	+	+
8	O121	+	+	O121	+	+
9	O145	+	+	O103	+	+
10	O118	+	+	O157:H7	+	+
11	O5	+	+	O103	+	+
12	Ount	+	+	O103	-	-
				Ount	+	+
13	O91	+	-	O113	+	-
	O26	-	+	O26	-	+

*Ount, O type untypable

Table 3.2: Collection and storage of sample enrichment broth

Purpose	Enrichment Time	Volume (mL)	Storage Temperature
GENE-UP PCR detection	8 h, 10 h	5	4°C
GDS PCR detection	8 h	5	4°C
BioRad PCR detection	10 h	5	4°C
CSU Culture Confirmation	10 h	10	4°C
Storage for bioMérieux	8 h, 10 h	20	-20°C

Table 3.3: Potential positive sample numbers generated by GENE-UP-10 h, BioRad-10 h and GDS-8 h for the detection of Top 6 STEC and *E. coli* O157:H7

	GENE-UP-10hr		**BioRad-10 h**		**GDS-8 h**	
	Top 6 STEC	O157:H7	Top 6 STEC	O157:H7	Top 6 STEC	O157:H7
S1-S50	13	1	19	10	12	10
S51-S194	34	10	62	42	31	9

Table 3.4: Results for Top 6 STEC detection and culture isolation in S1-S50 using the GENE-UP, NM-EHEC, BioRad IQ-check PCR VirX MLG, and GDS PCR screening methods

		GENE-UP-8 h		GENE-UP-10 h		NM-EHEC-10 h		BioRad-10 h		GDS-8 h	
	Culture	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg
Positive	14	12	2	12	2	11	3	14	0	12	2
Negative	1	1	0	1	0	0	1	1	0	0	1
Total	15	13	2	13	2	11	4	15	0	12	3

* Pos: Positive; Neg: Negative

Table 3.5: Comparative evaluation of the GENE-UP, NM-EHEC, BioRad IQ-check PCR VirX MLG, and GDS PCR screening detection methods for Top 6 STEC against the culture confirmed 10 h-enrichment of S1-S50

	GENE-UP-8 h		GENE-UP-10 h		NM-EHEC-10 h		BioRad-10 h		GDS-8 h	
	%	([d]LCI, [e]UCI)	%	([d]LCI, [e]UCI)	%	([d]LCI, [e]UCI)	%	([d]LCI, [e]UCI)	%	([d]LCI, [e]UCI)
Sensitivity	85.7	(56.2, 97.5)	85.7	(56.2, 97.5)	78.6	(48.8, 94.3)	100.0	(73.2, 100)	85.7	(56.2, 97.5)
Specificity	--	--	--	--	100.0	(5.5, 100.0)	--	--	100.0	(5.5, 100.0)
False-positive rate	--	--	--	--	0.0	(0.0, 94.5)	--	--	0.0	(0.0, 94.5)
False-negative rate	14.3	(2.5, 43.8)	14.3	(2.5, 43.8)	21.4	(5.7, 51.2)	0.0	(0.0, 26.8)	14.3	(2.5, 43.8)
[a]PPV	92.3	(62.1, 99.6)	92.3	(62.1, 99.6)	100.0	(67.9,100.0)	93.3	(66.0, 99.7)	100.0	(69.9, 100)
[b]NPV	--	--	--	--	25.0	(1.3, 78.1)	[c]NA	(NA, NA)	33.3	(1.8, 87.5)
Overall accuracy	80.0	(51.4, 94.7)	80.0	(51.4, 94.7)	80.0	(51.4, 94.7)	93.3	(66.0, 99.7)	86.7	(58.4, 97.7)

-- data were not calculated because the number of true negative was zero

[a] PPV: Positive Predictive Value

[b] NPV: Negative Predictive Value

[c] NA: the value is undefined when the denominator is zero

[d] LCI, Lower Confidence Interval

[e] UCI, Upper Confidence Interval

Table 3.6: Results for *E. coli* O157:H7 detection and culture isolation in S1-S50 using the GENE-UP, NM-EHEC, BioRad IQ-check PCR VirX MLG, and GDS PCR screening methods

		GENE-UP-8 h		GENE-UP-10 h		NM-EHEC-10 h		BioRad-10 h		GDS-8 h	
	Culture	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg
Positive	9	1	8	1	8	1	8	8	1	8	1
Negative	6	0	6	0	6	0	6	1	5	2	4
Total	15	1	14	1	14	1	14	9	6	10	5

* Pos: Positive; Neg: Negative

Table 3.7: Comparative evaluation of the GENE-UP, NM-EHEC, BioRad IQ-check PCR VirX MLG, and GDS PCR screening detection methods for *E. coli* O157:H7 against the culture confirmed enrichment of S1-S50

	GENE-UP-8 h		GENE-UP-10 h		NM-EHEC-10 h		BioRad-10 h		GDS-8 h	
	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)
Sensitivity	11.1	(0.6, 49.3)	11.1	(0.6, 49.3)	11.1	(0.6, 49.3)	88.9	(50.7, 99.4)	88.9	(50.7, 99.4)
Specificity	100.0	(51.7, 100.0)	100.0	(51.7, 100.0)	100.0	(51.7, 100.0)	83.3	(36.5, 99.1)	66.7	(24.1, 94.0)
False-positive rate	0.0	(0.0, 48.3)	0.0	(0.0, 48.3)	0.0	(0.0, 48.3)	16.7	(0.9, 63.5)	33.3	(6.0, 75.9)
False-negative rate	88.9	(50.7, 99.4)	88.9	(50.7, 99.4)	88.9	(50.7, 99.4)	11.1	(0.6, 49.3)	11.1	(0.6, 49.3)
[a]PPV	100.0	(5.5, 100.0)	100.0	(5.5, 100.0)	100.0	(5.5, 100.0)	88.9	(50.7, 99.4)	80.0	(44.2, 96.5)
[b]NPV	42.9	(18.8, 70.4)	42.9	(18.8, 70.4)	42.9	(18.8, 70.4)	83.3	(36.5, 99.1)	80.0	(29.9, 98.9)
Overall accuracy	46.7	(22.3, 72.6)	46.7	(22.3, 72.6)	46.7	(22.3, 72.6)	86.7	(58.4, 97.7)	80.0	(51.4, 94.7)

[a] PPV: Positive Predictive Value
[b] NPV: Negative Predictive Value
[c] LCI, Lower Confidence Interval
[d] UCI, Upper Confidence Interval

Table 3.8: Results for Top 6 STEC detection and culture isolation in S51-S194 using the GENE-UP, NM-EHEC, BioRad IQ-check PCR VirX MLG, and GDS PCR screening methods

	Culture	GENE-UP-8 h		GENE-UP-10 h		NM-EHEC-10 h		BioRad-10 h		GDS-8 h	
		*Pos	*Neg	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg
Positive	34	31	3	32	2	32	2	31	3	28	6
Negative	8	1	7	2	6	2	6	4	4	3	5
Total	42	32	10	34	8	34	8	35	7	31	11

* Pos: Positive; Neg: Negative

Table 3.9: Comparative evaluation of the GENE-UP, NM-EHEC, BioRad IQ-check PCR VirX MLG, and GDS PCR screening detection methods for Top 6 STEC against the culture confirmed enrichment of S51-S194

	GENE-UP-8 h		GENE-UP-10 h		NM-EHEC-10 h		BioRad-10 h		GDS-8 h	
	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)
Sensitivity	91.2	(75.2, 97.7)	94.1	(78.9, 99.0)	94.1	(78.9, 99.0)	91.2	(75.2, 97.7)	82.4	(64.8, 92.6)
Specificity	87.5	(46.7, 99.3)	75.0	(35.6, 95.5)	75.0	(35.6, 95.5)	50.0	(17.4, 82.6)	62.5	(25.9, 89.8)
False-positive rate	12.5	(0.7, 53.3)	25.0	(4.5, 64.4)	25.0	(4.5, 64.4)	50.0	(17.4, 82.6)	37.5	(10.2, 74.1)
False-negative rate	8.8	(2.3, 24.8)	5.9	(1.0, 21.1)	5.9	(1.0, 21.1)	8.8	(2.3, 24.8)	17.6	(7.4, 35.2)
[a]PPV	96.9	(82.0, 99.8)	94.1	(78.9, 99.0)	94.1	(78.9, 99.0)	88.6	(72.3, 96.3)	90.3	(73.1, 97.5)
[b]NPV	70	(35.4, 91.9)	75.0	(35.6, 95.5)	75.0	(35.6, 95.5)	57.1	(20.2, 88.2)	45.5	(18.1, 75.4)
Overall accuracy	90.5	(76.5, 96.9)	90.5	(76.5, 96.9)	90.5	(76.5, 96.9)	83.3	(68.0, 92.5)	78.6	(62.8, 89.2)

[a] PPV: Positive Predictive Value
[b] NPV: Negative Predictive Value
[c] LCI, Lower Confidence Interval
[d] UCI, Upper Confidence Interval

Table 3.10: Results for *E. coli* O157:H7 detection and culture isolation in S51-S194 using the GENE-UP, BioRad IQ-check PCR VirX MLG, and GDS PCR screening methods

		GENE-UP-8 h		GENE-UP-10 h		NM-EHEC-10 h		BioRad-10 h		GDS-8 h	
	Culture	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg	*Pos	*Neg
Positive	11	8	3	10	1	10	1	11	0	7	4
Negative	42	0	42	0	42	0	42	8	34	2	40
Total	53	8	45	10	43	10	43	19	34	9	44

* Pos: Positive; Neg: Negative

Table 3.11: Comparative evaluation of the GENE-UP, BioRad IQ-check PCR VirX MLG, and GDS PCR screening detection methods for *E. coli* O157:H7 against the culture confirmed enrichment of S51-S194

	GENE-UP- 8 h		GENE-UP-10 h		NM-EHEC-10 h		BioRad-10 h		GDS-8 h	
	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)	%	([c]LCI, [d]UCI)
Sensitivity	72.7	(39.3, 92.7)	90.9	(57.1, 99.5)	90.9	(57.1, 99.5)	100.0	(67.9, 100)	63.6	(31.6, 87.6)
Specificity	100.0	(89.6, 100.0)	100.0	(89.6, 100.0)	100.0	(89.6, 100)	81.0	(65.4, 90.9)	95.2	(82.6, 99.2)
False-positive rate	0.0	(0.0, 10.4)	0.0	(0.0, 10.4)	0.0	(0.0, 10.4)	19.0	(9.1, 34.6)	4.8	(0.8, 17.4)
False-negative rate	27.3	(7.3, 60.7)	9.1	(0.5, 42.9)	9.1	(0.5, 42.9)	0.0	(0.0, 32.1)	36.4	(12.4, 68.4)
[a]PPV	100.0	(59.8, 100.0)	100.0	(65.5, 100)	100.0	(65.5, 100.0)	57.9	(34.0, 78.9)	77.8	(40.2, 96.1)
[b]NPV	93.3	(80.7, 98.3)	97.7	(86.2, 99.9)	97.7	(86.2, 99.9)	100.0	(87.4, 100.0)	90.9	(77.4, 97.0)
Overall accuracy	94.3	(83.4, 98.5)	98.1	(88.6, 99.9)	98.1	(88.6, 99.9)	84.9	(71.9, 92.8)	88.7	(76.3, 95.3)

[a] PPV: Positive Predictive Value
[b] NPV: Negative Predictive Value
[c] LCI, Lower Confidence Interval
[d] UCI, Upper Confidence Interval

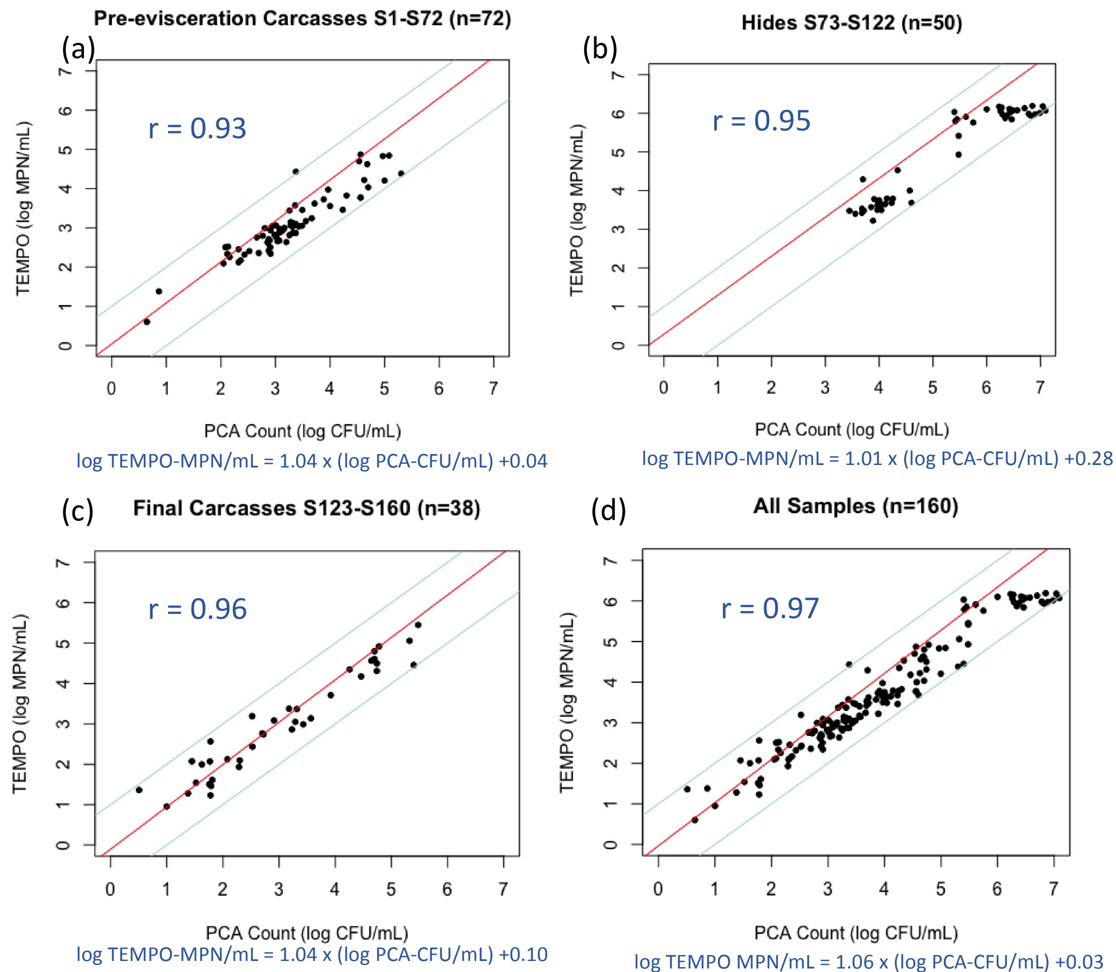

Figure 2.1: Scatterplots demonstrating the correlation between the log-transformed automated MPN (TEMPO) and traditional method (PCA plating) total aerobic mesophilic microflora counts for (a) pre-evisceration carcasses (b) hides (c) post-chill final carcasses and (d) all samples.

The best fit linear regression line (red color) is bounded by curves (blue color) representing 1 log difference intervals. The blue color curves (y=x+1, y=x-1) set an area and the differences between the two methods were 1) <1 log if the dots fell within this area, 2) =1 log if the dots fell onto the two lines and 3) >1 log if the dots fell outside this area.

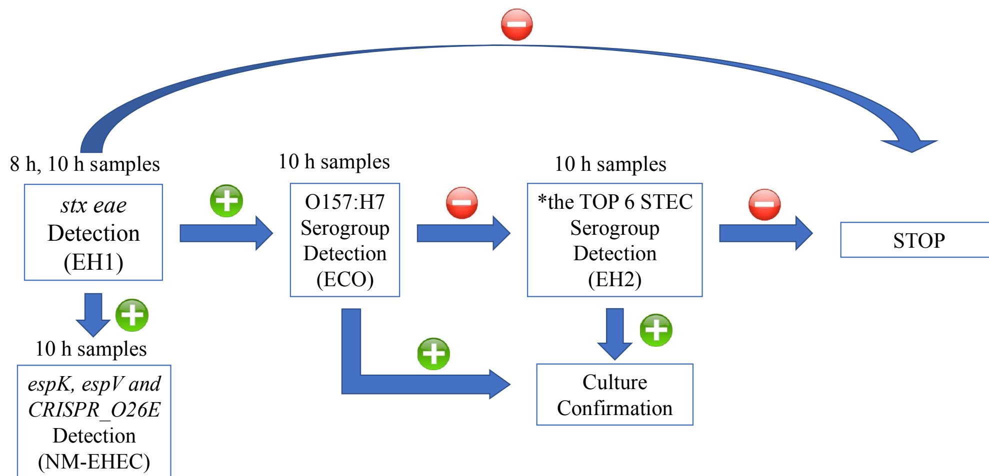

Figure 3.1: Workflow of GENE-UP analysis for S51-S194 at CSU (*the Top 6 STEC is defined as *E. coli* belonging to serogroups O26, O45, O103, O111, O121, or O145 that possess both *stx* and *eae* virulence genes). The EH1 assay was performed on all 8 h and 10 h BPW samples, and the rest of the assays (ECO, EH2 and NM-EHEC) were performed on 10 h BPW samples.

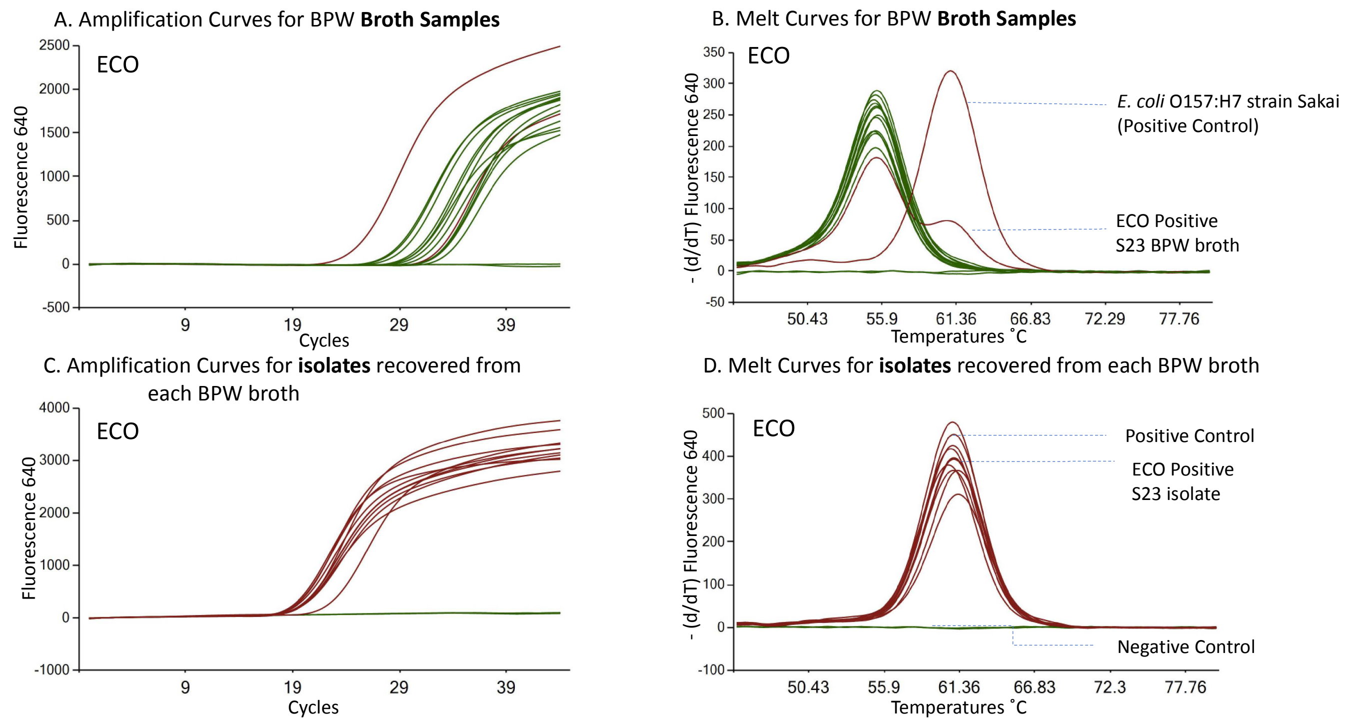

Figure 3.2: Representative amplification and melt curves for EH1-positive BPW broth samples (A, B), and isolates recovered from these BPW broth samples (C, D), using the ECO assay for *E. coli* O157:H7 detection. The Tm peaks in (B) show one BPW sample to be *E. coli* O157:H7 positive (red) and the other samples as negative (green), while the Tm peaks in (D) showed all isolates are *E. coli* O157:H7 positive. These results indicate that the shift in Tm resulted in 8 false negatives for GENE-UP detection for *E. coli* O157:H7 in S1-S50.

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