DEVELOPMENT OF AN INNOVATIVE DETECTION TECHNOLOGY FOR

ESCHERICHIA COLI O157:H7

By

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Escherichia coli O157:H7 detection in food is conducted mainly by DNA/PCR, immunoassay or conventional methods. However, all the methods require multiple incubation steps. Antibiotic and isolation agars were found as the main factors that lead to false-positive results. An improved rapid detection method was developed by decreasing detection time and enhancing easiness of detection without the need for any analytical instrumentation. A combination of selective ingredients and temperature was utilized to allow the growth of *Escherichia coli* O157:H7 in the detection. The detection method minimized the effects of the main false positive bacteria, *Pseudomonas* spp. and *Enterobacter* spp. The sensitivity, specificity and accuracy of the 24h detection method in foodstuffs were 96.2%, 99.6% and 97.0%, respectively when the original inoculation was 10-100cfu/g in food. This method can be utilized to detect *Escherichia coli* O157:H7 in foodstuffs more rapidly, economically and conveniently when compared to the methods that are currently used.

Key words: *Escherichia coli* O157:H7, false positive, 24h rapid detection

DEDICATION

I would like to dedicate this research to my parents, my grandparents and my boyfriend.

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CHAPTER I

INTRODUCTION

Escherichia coli have been used as markers for water contamination by the U.S. Public Health Service since 1914 (Tortorello, 2003). Although most *Escherichia coli* strains are harmless, some are opportunistic pathogenic bacteria due to their flagella. *Escherichia coli* O157:H7 formed by this process was the result of the shiga toxin gene transformed from *Shigella* to *Escherichia coli* (Brüssow et al., 2004). Ruminants and *Escherichia coli* O157:H7 are considered as commensals (Griffin, 1991). *Escherichia coli* O157:H7 can be considered as a transient member of the bovine microbiota and colonize in the recto-anal junction mucosa of cattle (Cobbold, 2007). From 2006 to 2011, the Centers for Disease Control and Prevention reported five *Escherichia coli* O157:H7 outbreaks that were associated with beef products in 29 states (CDC, 2011).*Escherichia coli* O157:H7 is not only able to colonize on ruminants, but also environmental reservoirs (Hancock, 1997). Water troughs for cattle are long-term environmental reservoirs of *Escherichia coli* O157:H7 and induce the transmission of *Escherichia coli* O157:H7 among cattle because *Escherichia coli* O157:H7 is able to survive for more than six months (LeJeune, 2001). *Escherichia coli* O157:H7 has been detected in some rivers, such as the Ganga river in India (Hamner, 2007) and Oldman river in Canada (Johnson, 2003). The fact that it survives in water for long times not only directly induces drinking water-infections, but also outbreaks related with seafood. Recently, in India, *Escherichia coli* O157:H7 was isolated from retail shrimp (Surendraraj, 2010). Moreover, at least one

outbreak of *Escherichia coli* O157:H7 was reported to probably been associated with catfish. In 2008, farm-raised catfish were assessed, and identified as a potential hazard (McCoy, 2011). *Escherichia coli* O157:H7 may have penetrated through the mucus of catfish (Suhalim, 2007).

To enhance screening for *Escherichia coli* O157:H7, a rapid and sensitive detection method could be developed for the food and allied industries. This method could prevent human and economic losses, and control the transmission of *Escherichia coli* O157:H7 from source to human or from one source to another source (Gould, 2005). Many methods can detect and identify *Escherichia coli* O157:H7. Deisingh (2004) compared the limitations among conventional plating-culture methods, biochemical methods, antibody-antigen immunological detection, PCR based detection, biosensors and GFP fluorescence. Currently, USDA and FDA have standard methods that mainly focus on conventional plating-culture methods and PCR-based detection since the former has low detection limits and low costs while the latter is highly sensitive and accurate (BAM, 2011; FSIS 2010). However, all of these methods require multiple incubation steps that enrich *Escherichia coli* O157:H7, and allow the growth of other background microorganisms such as Pseudomonas, generic *Escherichia coli* and *Enterobacter*, but they may still lead to false-positive results. No detection method for *Escherichia coli* O157:H7 is currently available that can minimize false positive results and improve sensitivity while decreasing detection time and easing mode of detection.

The objective of this study was to develop and optimize a detection method to provide benefits to increase sensitivity, specificity and accuracy of conventional plating-culture methods, reduce total incubation time and potentially provide an on-site detection method that does not required any analytical instrumentation.

The objectives of the study were to:

1. Screen catfish (live at farms) for Escherichia coli O157:H7 using FDA/BAM standard methods.
2. Study factors which may affect sensitivity of detection methods.
3. Develop a rapid detection method for Escherichia coli O157:H7 in foodstuffs.

CHAPTER II

LITERATURE REVIEW

Escherichia coli

Introduction

Escherichia coli are gram-negative, non-spore forming, rod-shaped bacteria that were discovered in 1885. *Escherichia coli* have optimal growth at 37 ℃, and are capable of living on wide variety of substrates. *Escherichia coli* belong to the *Enterobacteriaceae* family since they grow in the intestine of humans and warm-blooded animals. *Escherichia coli* are used as indicators of fecal contamination or unsanitary processing because *Escherichia coli* are mainly abundant in human and animal feces. Although most *Escherichia coli* strains are harmless, they are opportunistic pathogenic bacteria. The reason is that *Escherichia coli* possess flagella. *Escherichia coli* can swim because it possesses many motile flagella, and these flagella can transfer DNA material from one to another. *Escherichia coli* O157:H7 is formed by this process; shiga toxin gene is transformed from *Shigella* to *Escherichia coli* (Brüssow et al., 2004).

Biochemical characteristics

Escherichia coli are facultative anaerobic bacteria that make use of carbohydrates by anaerobic fermentation or aerobic respiration. During respiration, carbohydrates are decomposed into ATP, carbon dioxide and water. However, during carbohydrate fermentation, mixed acids, such as lactate, succinate, ethanol, acetate and carbon dioxide

are produced. Based on reports, most of *Escherichia coli* strains are positive for sorbitol, melibiose, arabinose and glucose fermentation, while break down sucrose (EVRYLL, 1980). Meanwhile, *Escherichia coli* possess decarboxylase activity and produce amines that increase the pH value of the media. Almost 90% of *Escherichia coli* have L-lysine and L-ornithine decarboxylase. In decarboxylase medium, the pH value is always decreased first by fermentation of glucose, and then under the optimal pH value of decarboxylase activity, decarboxylation occurs (Gunnar, 1972). The optimal pH value of L-lysine decarboxylase is 6.0, and when the pH value is lower or higher than 6.0, the activity of decarboxylase is decreased (Gale, 1944). At a pH of 5.5, L-ornithine decarboxylase can rapidly increase the pH of the medium (Gunnar, 1972).

In addition, *Escherichia coli* strains have β-galactosidase and β-D-glucuronidase activity. The former is produced by the lac operon in order to decompose lactose. This characteristic is frequently applied in the field of molecular biology, such as a screening target modified bacteria by the X-gal that contains galactose. And β-D-glucuronidase activity is broadly used in the diagnosis of *Escherichia coli* by utilizing the fluorescent substance, 4-methylumbelliferyl-β-D-glucuronide (MUG) (Feng, 1992).

Pathogenic *Escherichia coli*

Pathogenic *Escherichia coli* refer to a group of *Escherichia coli* that can cause diarrheal disease, and are also referred to as diarrheagenic *Escherichia coli* (James, 1998). *Escherichia coli* are classified in foodborne diseases based on their different virulent characteristics and include as enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enterohemorrhagic(EHEC) (Levine, 1987).

ETEC can produce and introduce enterotoxins to intestinal cells by adhering and transmitting. There are two kinds of enterotoxins; heat-labile toxins (LTs) and heat-stable toxins (STs) (Cassels, 1995). EPEC can attach, efface and lesion adsorptive microvilli of intestinal cells by adherence factor and intimin protein, which causes diarrhea occurs due to malabsorption (Hicks, 1998). EHEC is similar to EPEC because both possess intestinal adherence factors that are encoded by the *eae* gene. The only difference is that the attachment and effacement lesions caused are in the different part of the intestines (Yu, 1992). In addition, EHEC can produce cytotoxin, *Shigella* dysenteriae I (Shiga) toxin (*Stx*) that could cause hemorrhagic colitis (HC) (Riley, 1983) and hemolytic uremic syndrome (HUS) (Karmali, 1983). The EIEC is able to invade and destroy intestinal cell tissue due to the coding of invasion genes (DuPont, 1971).

***Escherichia coli* O157:H7**

Pathogenesis

Escherichia coli O157:H7 were firstly recognized as pathogenic *Escherichia coli*, and classified as enterohemorrhagic *Escherichia coli* (EHEC) since infected patients by were reported to both of hemorrhagic colitis and hemolytic uremic syndrome (Karmali, 1983; Riley, 1983). The pathogenic factors of *Escherichia coli* O157:H7 are similar and to most EHEC, and mainly focus on shiga toxins, EAST1, enterohemolysin, intestinal adherence factors (James, 1998).

The major virulence factor is Shiga toxin (Stx). It inhibits protein synthesis by affecting EF-1-dependent amino acyl transfer ribonucleic acid (amino acyl-tRNA) binding (Brown, 1986). There are two major Shiga toxin and include Stx1 and Stx 2. Although *Escherichia coli* O157:H7 may express either one or both, some Stx2 variants

are more likely to induce cytotoxicity and cause hemolytic uremic syndrome when compared to Stx1 (James, 1998). In addition, *Escherichia coli* O157:H7 often have EAST1, enteroaggregative heat-stable enterotoxins, which are always encoded by the *ast* gene in EAEC (Savarino, 1996). This characteristic is used to identify *Escherichia coli* O157:H7. The *eae* gene is another type of virulence factor in *Escherichia coli* O157:H7. As characteristic of other EPEC, *Escherichia coli* O157:H7 can possess intimin protein that brings attachment and effaces lesions in the large tissues coded by the *eae* gene (James, 1998). Intimin plays an important role in the transmission of *Escherichia coli* O157:H7 because intimin negative *Escherichia coli* O157:H7 can colonize in the intestines and which makes it less likely to cause disease (Evelyna, 1998). Another virulence factor, enterohemolysin, can be coded by the *ehx* gene in the pO157 plasmid on nearly all of *Escherichia coli* O157:H7 and non-*Escherichia coli* O157:H7 (Beutin, 1994).

Biochemical characteristics

Unlike genetic *Escherichia coli* or other pathogenic *Escherichia coli*, *Escherichia coli* O157:H7 are not able to ferment sorbitol and do not possess β-D-glucuronidase activity. The β-D-glucuronidase is encoded by the *uid*A gene and appears in the *Enterobacteriaceae*, such as most *Escherichia coli* (Hartman, 1989), some *Shigella* and *Salmonella*. *Escherichia coli* O157:H7 also possesses the *uid*A gene (FENG, 1991), but in which one thymine is replaced for guanine at position 92 due to a base-pair mismatch (Feng, 1994). *Escherichia coli* O157: H^- is another EHEC that are different from *Escherichia coli* O157:H7 in mobility and glucuronidase activity based on gene mutation, but both are from one EPEC, more specifically *Escherichia coli* O55:H7.

In addition, different strains of *Escherichia coli* O157:H7 may be slightly different. Although *Escherichia coli* O157:H7 is resistant to acid (Francisco, 1997), different strains have different resistance levels to acids. When comparing ATCC 43889, ATCC 43895, it was shown that ATCC 43895 can survive with a lower decrease in of *Escherichia coli* counts at acidic condition (Miller, 1994).

Isolation, Detection and Identification of *Escherichia coli* O157:H7

In the industry, sensitive and reliable methods for detection of *Escherichia coli* O157:H7 are needed to detect and control the contamination of *Escherichia coli* O157:H7 prior to food consumption. Many methods can detect and identify *Escherichia coli* O157:H7.Deisinghand Thompson (2004) have compared the limitations among conventional planting-culture methods, biochemical methods, antibody-antigen immunological detection, PCR based detection, biosensors and GFP fluorescence. Currently, USDA and FDA publicize standard methods that mainly focus on conventional methods and PCR based detection because the former has low detection limits and cost, and the latter is highly sensitive with a short detection time (BAM, FSIS).

Enrichment Broth

Enrichment broth is always used prior to plating on selective agar in order to repair and enhance the growth of injured bacteria. With respect to the enrichment broth of *Escherichia coli* O157:H7, peptone, carbohydrate, sodium chloride and some buffering agents are normally included. However, the concentration and species of ingredients could be changed to increase the sensitivity of detection. Jassonet.al. (2009) 10 commercial enrichment broths of *Escherichia coli* O157:H7, and reported that all broths failed to recover at least one type of injured *Escherichia coli* O157:H7. In addition,

different food may show various sensitivities of detection in different enrichment broths. Lactose can enrich *Escherichia coli* O157:H7 better in radish sprout, but in ground beef the efficiencies of lactose and glucose are the same (Yukiko, 1999). However, no carbohydrate increases the sensitivity of detection for *Escherichia coli* O157:H7. Peptone water may be more effective and faster than other broths with carbohydrate (Stephen, 2001). Subsequently, for a higher requirement of accuracy, vancomycin, cefixime and cefsulodin are used to enhance the detection of *Escherichia coli* (Weagant, 1995). Vancomycin is a glycopeptide antibiotic that is effective against Gram-positive bacteria by inhibiting the synthesis of the cell wall. Cefixime as a third generation of cephalosporin antibiotics is effective against other *Enterobacteriaceae*, but has no inactivity against *Pseudomonas* (Brogen, 1989). In addition, another kind of cephalosporin, cefsulodin, is able to inhibit the growth of 50% *Pseudomonas* spp. due to a special affinity for them (Harold, 1984).

Isolation Agar

Sorbitol MacConkey agar is the most normal isolation agar for the detection of *Escherichia coli* O157:H7 due to the negative sorbitol fermentation of *Escherichia coli* O157:H7. In Sorbitol MacConkey agar, *Escherichia coli* O157:H7 shows up as colorless colonies, but other *Enterobacteriaceae*, such as generic *Escherichia coli* or other pathogenic *Escherichia coli*, appear pink because they can ferment sorbitol to produce lactic acid. Neutral red as the pH indicator of Sorbitol MacConkey agar and is expressed as pink at acidic pH values. Peptone is used to maintain the growth of *Escherichia coli* O157:H7, instead of sorbitol like other *Enterobacteriaceae*, in Sorbitol MacConkey agar, so colorless colonies appear (March, 1986). As the enrichment broth, Sorbitol

MacConkey agar is also enhanced by two additional antibiotics: cefixime and potassium-tellurite are used to inhibit other sorbitol non-fermenters, such as *Proteus* spp., *Providencia* spp., *Aeromonas* spp. and other sorbitol non-fermentation *Escherichia coli* (Zadik, 1993). However, *Pseudomonas* spp. can also grow on Sorbitol MacConkey agar and appear colorless (Cousin, 2000), so more modified agar methods have been developed and focus on chromogenic technology. In the Bacteriological Analytical Manual of the U.S. Food and Drug Administration, these agars use chromogenic selective agar, Rainbow® Agar O157 and R&F®*Escherichia coli* O157:H7 agar (BAM, 2009). All of them mainly apply chromogenic enzyme substrates associated with β-galactosidase and β-glucuronidase, and various colored colonies appear based on the different enzymatic characteristic of *Escherichia coli* O157:H7 and other bacteria (Anonymous, 2003; Restaino, 2003). In addition, similarly, Fujisawa utilized the fluorescent substance, 4-methylumbelliferyl-β-galactopyranoside and the β-galactosidase activity of *Escherichia coli* to eliminate other sorbitol non-fermentation bacteria, like *Pseudomonas* (Fujisawa, 2000).

Polymerase Chain Reaction

Polymerase chain reaction is used to detect bacteria since it can rapidly amplify a particular DNA sequence and then confirm the existence of a pathogen by comparing it with an objective DNA sequence (Mullis, 1983; Greisen, 1994). With respect to *Escherichia coli* O157:H7, the objective genes mainly focus on the gene related with biochemical characteristics and virulence factors, such as the *uid*A gene, *stx*1 gene, *stx*2 gene and the *eae*A gene. Because one gene may exist in different kinds of bacteria, multiplex polymerase chain reaction that co-amplifies multiple target genes provides

more sensitive and accurate results (Newton, 1997). Feng (2002) describes a multiplex polymerase chain reaction assay for detecting *Escherichia coli* O157:H7 by five virulence and trait genetic markers (*uid*A, *stx*1, *stx*2, *eae*A, and *ehx*A) while identifying STEC and other EHEC based on different combinations of genes. In addition, real-time polymerase chain reactions are used to detect bacteria because it can not only identify the target gene, but also quantify them by the time additional fluoresce probe (Heid, 1996). Currently, the standard methods of USDA and FDA utilize real-time polymerase chain reaction due to its more rapid and sensitive characteristics (USDA, BAM).

Normally, polymerase chain reaction is used as two formats. One polymerase chain reaction method is carried out after enrichment or even simple preparation and extraction by immune-magnetic separation or buoyant density centrifugation. Chapman (2001) reported that polymerase chain reaction with immune-magnetic separation is the most sensitive method when compared with culture and immunoassays for the detection of *Escherichia coli* O157:H7 in raw meat products. The combination of immunomagnetic separation and polymerase chain reaction possesses more rapid, specific, sensitive quantitative characteristics for the detection of *Escherichia coli* O157:H7, and enrichment step is unnecessary (Fu,2005). The other polymerase chain reaction method is associated with isolation agar. In the USDA standard method for the detection of *Escherichia coli* O157:H7 in meat products, Rainbow® Agar O157 and polymerase chain reactions are combined (BAM). Radu (2000) evaluated the combination of commercial isolation agar for *Escherichia coli* O157:H7 and polymerase chain reaction and reported that it can be used of screen and confirm *Escherichia coli* O157:H7 in 24h.

Biochemical Tests

Biochemical tests follow isolation by agar or confirmation by polymerase chain reactions to further differentiate *Escherichia coli* O157:H7 from other *Enterobacteriaceae* or to identify false-positive bacteria after confirmation through polymerase chain reaction. *Escherichia coli* O157:H7 is identified by biochemical tests after the differentiation of modified selective medium (Park, 2011). Kobayashi (2009) examined *Escherichia coli* O157:H7 by polymerase chain screening and then biochemical confirmation. Usually, the API system (BioMerieux, La) which includes 15 identification systems that can identify over 600 different species and is applied for the detection of bacteria based on the Bacteriological Analytical Manual of U.S. Food and Drug Administration (BAM, 2009)

Epidemiology of *Escherichia coli* O157:H7

In order to recognize the cause of disease and control the transmission of pathogenic bacteria and disease outbreaks, epidemiology is used to collect and analyze incidence, identify pathogens and related factors (Green, 2008). For *Escherichia coli* O157:H7, epidemiology mainly focuses on incidence, reservoir and transmission (James, 1998).

Incidence

Based on the data for the Foodborne Diseases Active Surveillance Network, incidence of *Escherichia coli* O157:H7 has decreased from 2 cases per 100,000 people in 1997 to 0.9 cases per 100,000 people (Food Net, 2010) since detection and investigation have been improved, preventing contaminations has been emphasized and the awareness on risk of *Escherichia coli* O157:H7 has been enhanced (CDC, 2010). However, once an

outbreak of *Escherichia coli* O157:H7 occurs, the aftereffects are serious. In 2010, there were 184 hospitalization-cases and 2 death-cases (Food Net, 2010). Meanwhile, after the outbreaks, the loss of economics is huge due to the food recall. As of March 22, 2011, 14 persons were infected with *Escherichia coli* O157:H7 from Palmyra Bologna which led to the recall of approximately 23,000 pounds of Lebanon bologna (CDC, 2011).

Additionally, *Escherichia coli* O157:H7 shows seasonal prevalence and a positive correlation among day length, ambient temperature and *Escherichia coli* O157:H7 prevalence (Tom, 2007). Barkocy-Gallagher G.A (2003) reported that the prevalence of *Escherichia coli* O157:H7 was increased from spring to summer, and then decreased to a minimal prevalence through winter when investigating beef processing plants.

Reservoir and Transmission

Ruminants and *Escherichia coli* O157:H7 are considered as commensals, and cattle are the primary reservoir of *Escherichia coli* O157:H7 (Griffin, 1991). *Escherichia coli* O157:H7 can be considered as a transient member of the bovine microbiota. It is able to colonize in the recto-anal junction mucosa of cattle (Cobbold, 2007) and is protected by host cell internalization of bovine rectal epithelial cells (Sheng, 2011). From 2006 to 2011, the Centers for Disease Control and Prevention reported that there were 5 *Escherichia coli* O157:H7 outbreaks associated with beef products that involved 29 states (CDC, 2011). In France, sixty-nine patients, including 17 cases of HUS were identified from infection with *Escherichia coli* O157:H7 in October 2005. Investigation into this outbreak of *Escherichia coli* O157:H7 indicated that frozen beef products was the source of *Escherichia coli* O157:H7 (King, 2009).

In addition to cattle, some other types of animals have been linked to the occurrence of *Escherichia coli* O157:H7, such as chicken, turkey, swine (Doane, 2007) and wild birds (Foster, 2006). *Escherichia coli* O157:H7 is able to colonize many species and environmental reservoirs (Hancock, 1997). The water troughs used for cattle are a long-term environmental reservoir of *Escherichia coli* O157:H7 since it induces the transmission of *Escherichia coli* O157:H7 among cattle. This is because *Escherichia coli* O157:H7 is able to survive for more than six months and infect calves (LeJeune, 2001). Farm water is treated as a medium of transmission within herds based on the observation of the long-term survival of *Escherichia coli* O157:H7 (McGee, 2002). Thus, some outbreaks of *Escherichia coli* O157:H7 may be related to the cross-contamination of water. In addition, *Escherichia coli* O157:H7 has been detected in some rivers including Ganga in India (Hamner, 2007) and the Oldman River in Canada (Johnson, 2003). Therefor *Escherichia coli* O157:H7 infections can occur from drinking water and outbreaks related with seafood. Recently, In India, one *Escherichia coli* O157:H7 was isolated from retail shrimp (Surendraraj, 2010). Moreover, some other seafood is also at risk. For example, at least one outbreak of *Escherichia coli* O157:H7 has been associated with catfish. In 2008, farm-raised catfish was assessed, and identified as a potential hazard (McCoy, 2011) since *Escherichia coli* O157:H7 may penetrate through the mucus of catfish, and then contaminate catfish products (Rico R. Suhalim, 2007).

Public Health Consideration

Escherichia coli O157:H7 is a major public health consideration due to its ability to infect persons of all ages and colonize in the ruminants, low infectious dose but high

risk, various acid tolerances, and uncontrolled prevalence in food (Buchanan, 1997, CDC).

CHAPTER III

FACTORS AFFECTING DETECTION OF *ESCHERICHIA COLI* O157:H7

Abstract

Escherichia coli detection has focused mainly on DNA/PCR or immunoassay methods. An investigation of *Escherichia coli* O157:H7 in catfish farms and processing plants enabled us to evaluate the possible prevalence of *Escherichia coli* O157:H7 on catfish. However, a reliable and accurate test for the presence of *Escherichia coli* O157:H7 in catfish has not been reported. Based on the survey of *Escherichia coli* O157:H7 in catfish farms and catfish processing plants, *Escherichia coli* O157:H7 was not detected in the summer, fall or winter of 2010. *Pseudomonas* spp. and generic *Escherichia coli* were the main false positive bacteria isolated, resulting in many false positives that resulted in increased detection times and effort. The factors that may lead to decreased number of false positives and detection time were attributed. There were enrichment media types, antibiotic in the enrichment media and isolation agars. It was found that enrichment types did not affect the detection for *Escherichia coli* O157:H7, but antibiotic added in the enrichment would decrease the sensitivity of detection whatever isolation agars followed. In addition, different isolation agars were found there were different sensitivities for *Escherichia coli* O157:H7. Sorbitol MacConkey agar has a low sensitivity (22%) because of a high number of false positives, but Rainbow® agar O157 (100%) can compete with direct multiplex PCR when enrichment without antibiotic.

Introduction

Escherichia coli are used as indicators of fecal contamination or unsanitary processing since *Escherichia coli* are not only abundant in human and animal feces, but also opportunistic pathogenic bacteria such as *Escherichia coli* O157:H7due to flagella transformation (Ewing, 1996; Brüssow et al., 2004).*Escherichia coli* O157:H7 can cause both hemorrhagic colitis and hemolytic uremic syndrome(Karmali, 1983; Riley, 1983), and it frequently possesses EAST1, intimin protein, shiga toxin and enterohemolysin. EAST1 is an enteroaggregative heat-stable enterotoxin (Savarino, 1996). Intimin protein can bring attachment and effacement lesions in large tissues (James, 1998).Shiga toxins (Stx) inhibit protein synthesis by affecting EF-1-dependent aminoacyl transfer ribonucleic acid (aminoacyl-tRNA) binding (Brown, 1986) and enterohemolysin can be coded by the *ehx* gene in the pO157 plasmid (James, 1998). Thus, the number of cases per 100,000 people has been reduced from 2.0 to 0.9 (Food Net, 2010) because of increased research and improved detection method. However, once *coli* O157:H7 is ingested by humans, it causes serious health problems and large economic losses.

Cattle are the primary reservoirs of *Escherichia coli* O157:H7, but chicken, turkey, swine and wild birds also have been linked with the occurrence of *Escherichia coli* O157:H7 (Griffin, 1991; Doane, 2007; Foster, 2006). Meanwhile, water is a long-term environmental reservoir where *Escherichia coli* O157:H7 may survive and contaminate foodstuffs. *Escherichia coli* O157:H7 has been isolated from some rivers (Hamner, 2007; Johnson, 2003), so it can also affect aquaculture products, such as retail shrimp (Surendraraj, 2010). In 2008, farm-raised catfish were one of the foods associated with an outbreak of *Escherichia coli* O157:H7 (McCoy, 2011). *Escherichia coli* O157:H7 may penetrate through the mucus of catfish, and then contaminate catfish products (Suhalim,

2007). However, many references reported the observance of *Escherichia coli* O157:H7 in catfish and mainly focus on the quantification of *Escherichia coli* and total coliforms (McCaskey, 1998).

Currently, USDA and FDA have standardized the detection method for *Escherichia coli* O157:H7by conventional plating-culture methods and PCR based detection (BAM, FSIS), but there is no report that explains the efficiency of the methods in the detection of *Escherichia coli* O157:H7. The ingredients in the enrichment media and isolation agar, like peptone, carbohydrates and antibiotics are not always helpful for the growth of *Escherichia coli* O157:H7 in different product matrixes. For low risk sources, such as catfish, a highly selective enrichment and isolation procedure may inhibit the recovery of *Escherichia coli* O157:H7, and even inhibit their growth.

Thus, the objective of this study was to analyze the detection sensitivity of *Escherichia coli* O157:H7 on catfish and studying the factors that influence detection of *Escherichia coli* O157:H7procedure, such as, antibiotic in enrichment and enrichment types, isolation agars.

Materials and Methods

Bacterial Strains

Four *Escherichia coli* O157:H7 strains were studied. Three strains ATCC43890, ATCC43895 and ATCC35150 were provided by the Department of Food Science, Nutrition and Health Promotion, Mississippi State University and one provided by the United States Department of Agriculture (Starkville, MS). All of the strains were maintained at -20 ℃ in Trypticase Soy Broth (TSB) (Becton, Dickinson and Company, Sparks, MD).

Cultural Media and Chemicals

Trypticase Soy Broth (Becton, Dickinson and Company), Modified Tryptone Soya Broth (Oxoid Ltd., Basingstoke), Brain Heart Infusion Broth (Becton, Dickinson and Company) and Buffered Peptone Water (Oxoid Ltd., Basingstoke) were used as enrichment broth in this study. V.C.C Supplement containing 8.0 mg/L vancomycin, 10.0 mg/L cefsulodin and 0.05mg/L cefixime were prepared and added to the enrichment broth (Lab M Limited). In addition, two kinds of isolation agar, Sorbitol-MacConkey Agar (Becton, Dickinson and Company) and Rainbow® Agar O157 (Biolog, Hayward), one antibiotic added in isolation agar, CT supplement including cefixime and tellurite (Oxoid Ltd., Basingstoke) and two fluorescent substances, 4-methylumbelliferyl-beta-D-galactopyranoside and 4-methylumbelliferyl-bata-D-xylopyranoside (Acros Organics, Thermo Fisher Scientific) were prepared.

Survey on *Escherichia coli* O157:H7 in Catfish

Sampling, Enrichment and Isolation of Escherichia coli O157:H7

The samples were collected from whole catfish skin, water and sediment in the ponds of six catfish farms. The methods of sampling, enrichment, isolation and identification of *Escherichia coli* O157:H7 from catfish were based on the Bacteriological Analytical Manual of the U.S. Food and Drug Administration (BAM, 2009) with some modifications. At each catfish farm, the whole catfish was collected randomly and then immediately aseptically rinsed with 225mL of 0.1% buffered peptone water in a sterile bag. Ponds' water and sediment were also collected and kept in separate sterile bags. After transporting to the laboratory, 25 g of each rinsate was weighed and

added to 225 mL of EHEC enrichment broth. Meanwhile 25 g of pond's water or sediment was also diluted in 225 mL of EHEC enrichment broth.

V.C.C Supplement containing 8.0 mg/L vancomycin, 10.0 mg/L cefsulodin and 0.05mg/L cefixime were added and mixed gently before all the collected samples were incubated at 37°Cfor 24 h for the enrichment of *Escherichia coli* O157:H7. After incubation, a loop of sample was streaked on Sorbitol-MacConkey agar containing CT antibiotic supplement (cefixime and tellurite), and then agars were incubated at 37°Cfor 24 h for isolation of *Escherichia coli* O157:H7.

Identification of Escherichia coli O157:H7 by PCR

Presumptive colorless colonies from Sorbitol-MacConkey agar were cultured in Trypticase Soy Broth at 37°C for 24 h and then 1 mL of the cultured Trypticase Soy Broth was centrifuged at 10000×g for 2 min. The supernatant was removed and 50µL deionized distilled water was added. After homogenizing, the suspension was boiled for 5min and then centrifuged at 10000×g for 2min (Gussow, 1989). Supernatant (2µL) as a template was added to pre-mixed PCR tubes containing 12.5µL 2×Go*Taq*® Green Master Mix (Promega, Madison, WI), 5µL primer mixtures which included forward primers and reverse primers of each *uid*A, *ehx*A, *stx*1,*stx*2 and *eae*A (Feng, 2002) and 5.5µL Go*Taq*® deionized distilled water (Promega, Madison). The DNA amplifications were performed in the Master cycler gradient machine (Eppendof, New York City). Based on the activity of DNA polymerase and the melting temperature (Tm) of each primer, DNA was denatured at 95°Cfor 5min, and then 30 cycles of amplifications of target DNA pieces followed which included denaturation at 94°Cfor 30s, annealing of primers at 60°Cfor 30s and extension of primers at 72°Cfor 30s. After amplifications, 5min extension at 72°C was

performed, and then final PCR products were held at 4℃. PCR products were separated on a 2% agarose gel (ICN Biomedicals, Inc., Aurora, OH) in 0.5×Tris-acetic -EDTA buffer (TAE, pH 8.0) containing ethidium bromide using submarine electrophoresis system at 135V for 20min (Mupid®exU, Advance), and finally were photographed in a compact digimage system (Major Science, Saratoga). Based on the Bacteriological Analytical Manual (BAM, 2009) and FSIS procedure for the detection of *Escherichia coli* O157:H7 (FSIS, 2008), PCR was also performed. EHEC enrichment broth (1 mL) was treated directly without culturing on isolation agar, and then the procedure of multiplex PCR identification of *Escherichia coli* O157:H7 was performed as described before (BAM, 2009).

Survey on Enrichment and Antibiotic on Growth of *Escherichia coli* O157:H7

Effect of Enrichment on Growth of Escherichia coli O157:H7

Escherichia coli O157:H7 was inoculated in Trypticase Soy Broth at 37℃for 24 h, and then diluted in 0.1% buffered peptone water to obtain target concentrations of *Escherichia coli* O157:H7. The effect of different enrichment media on the growth of *Escherichia coli* O157:H7 were tested through the relationship between the numbers of viable *Escherichia coli* O157:H7 cells in the media and incubation times. *Escherichia coli* O157:H7 (less than 10 cfu/ mL) were incubated in different enrichment media separately at 37℃, and then enrichment cultures were collected at 6h, 8h, 10h, 12h and 14h respectively. The number of viable *Escherichia coli* O157:H7 cells in the media were estimated through optical density (O.D.) which was measured by spectrophotometer (UV-1201, Shimadzu, Kyoto) at 600nm.

Effect of V.C.C Supplement on the Growth of Different Strains of Escherichia coli O157:H7

Escherichia coli O157:H7 (less than 10 cfu/mL) was enriched in the modified Tryptone Soya Broth with and without V.C.C supplement at 37°C. Enrichment cultures were collected at 6h, 8h, 10h, 12h and 14h, and the optical density was measured using a spectrophotometer at 600nm.

Effect of Isolation Agar after Enrichment with or without antibiotic on Sensitivity of Detection of *Escherichia coli* O157:H7

Escherichia coli O157:H7 were added to catfish fillet (25g). The inoculated catfish samples were incubated in modified Tryptone Soya Broth with and without V.C.C supplement antibiotics. After incubation, 1 mL enrichment media were tested directly by multiplex PCR as control. Meanwhile, a loop of enrichment culture was streaked on the five types of isolation agar, Sorbitol-MacConkey Agar with CT supplement (C), Rainbow® Agar O157 with and without CT supplement (R and CR), Sorbitol-MacConkey agar with 4-Methylumbelliferyl-beta-D-galactopyranoside (D1) and Sorbitol-MacConkey agar with 4-Methylumbelliferyl-beta-D-xylopyranoside (D2). Presumptive *Escherichia coli* O157:H7 colonies also need be confirmed by multiplex PCR. Sensitivity was calculated by the following formula (Eijkelkamp 2009):

Sensitivity% = number of true positives/number of true inoculated tests.

The sensitivities were compared among six detection methods after enrichment with and without antibiotic.

Statistical analysis

A split plot design within completely randomized design was used to research the sensitivity of detection under different enrichment broths and isolation agars. On

enrichment agar, with and without antibiotic (whole plot), five isolation agars (subplot) were used to isolate *Escherichia coli* O157:H7 after enrichment. The experiment was replicated 3 times. The sensitivities were calculated, and then results were analyzed using PROC GLM in SAS (SAS 9.2, SAS Institute Inc.). When significant differences in sensitivity ($P<0.05$) occurred among different enrichment broths and/or isolation agars, the Fisher's least significant difference test was used to determine differences in means.

Results

Survey on *Escherichia coli* O157:H7 in Catfish

The survey of *Escherichia coli*O157 in the catfish farms and plants indicated that no *Escherichia coli* O157:H7 was found in summer and fall of 2010 (Table 1). Whenever, the incidence of generic *Escherichia coli* isolated or present on the plate, there were presumptive *Escherichia coli* O157:H7 also present. In addition, a high number of presumptive *Escherichia coli* O157:H7 were shown on Sorbitol-MacConkey agar, but all presumptive colorless colonies on the Sorbitol-MacConkey agar were confirmed as false positive colonies by PCR and identified as *Pseudomonas* spp. and *Escherichia coli* by API20E, except for non-detectable bacteria. This confirmed that catfish is low risk for *Escherichia coli* O157:H7. McCaskey (1998) reported no *Escherichia coli* O157:H7 in 220 catfish fillets collected during four seasons in one year. Some generic *Escherichia coli* showed slow sorbitol-fermentation, so no pink color appeared on the sorbitol-MacConkey agar. Another false positive bacterium, *Pseudomonas* spp. also cannot utilize sorbitol as the energy source, so they grow on the agar by metabolizing protein and are colorless, just like *Escherichia coli* O157:H7 (Cousin, 2000). The API20E revealed that

Pseudomonas spp. only possessed argine decarboxylase; whereas there are all three decarboxylases, argine, lysine and ornithine decarboxylase in *Escherichia coli* O157:H7.

Effect of Enrichment and Antibiotic on Growth of *Escherichia coli* O157:H7

The relationship between the numbers of viable *Escherichia coli* O157:H7 cells and the optical density are shown in Table 2. The sensitive zone by optical density is between 0.010 and 0.999 which is equivalent to 10^5 to 10^9 viable *Escherichia coli* O157:H7 cells per milliliter. The effect of enrichment media on the growth of *Escherichia coli* O157:H7 is shown in Figure 1. Because the optical density was less than 0.010 before 6 hours, the results were recorded starting at the sixth hour. At the beginning of incubation at 37℃, there was no difference among different enrichment media.

The effect of vancomycin, cefsulodin and cefixime on the growth of *Escherichia coli* O157:H7 was shown in Figure 2. After optical density was increased to the sensitive zone, 10^5 cells were formed in one milliliter. The growth of *Escherichia coli* O157:H7 in the Modified Tryptone Soya Broth without the antibiotic was better than with the antibiotic at 37℃.

Effect of Isolation Agar after Enrichment with or without antibiotic on Sensitivity of Detection of *Escherichia coli* O157:H7

The effect of isolation agar on the detection of *Escherichia coli* O157:H7 only focused on 1-10 cfu/g because unstable detection occurred under a lower initial number of cells. After inoculation, *Escherichia coli* O157:H7were confirmed from enrichment broth by Multiplex PCR directly.100% *Escherichia coli* O157:H7 in catfish without antibiotic can be detected by PCR, and 66% *Escherichia coli* O157:H7can be detected in enrichment with antibiotic. After culturing on the isolation agars, the growth of

Escherichia coli O157:H7 was not 100%, so sensitivity of detection was not the same as direct Multiplex PCR from enrichment. This is because false positives grow on isolation agar, especially, Sorbitol MacConkey agar.

There was no interaction between enrichment and agar ($P>0.05$). Enrichment with antibiotic could inhibit the growth of *Escherichia coli* O157:H7 (Figure 2) and had a lower sensitivity than enrichment without antibiotic ($P<0.05$) (Figure 3). Rainbow® Agar O157 with and without CT-supplement showed a higher sensitivity (72%) than CT-Sorbitol MacConkey agar with and without fluorescent substances ($P<0.05$). However, fluorescent substances, 4-Methylumbelliferyl-beta-D-xylopyranoside (D1) and 4-methylumbelliferyl-beta-D-galactopyranoside (D2) did not change the sensitivity of detection for *Escherichia coli* O157:H7 in Sorbitol MacConkey agar. 4-Methylumbelliferyl-beta-D-xylopyranoside can distinguish *Escherichia coli* O157:H7 with other bacteria under the UV light, so it has the same function as sorbitol in the Sorbitol MacConkey agar. 4-methylumbelliferyl-beta-D-galactopyranoside is able to distinguish between *Escherichia coli* and other bacteria under the UV light, so it can decrease false positive results, like *Pseudomonase*. However, in foodstuffs, the load of generic *Escherichia coli* is high, and then most of them containβ-galactosidase and produce fluorescence. The source of fluorescence cannot be distinguished, so sensitivity cannot be increased.

Discussion

Results confirmed catfish is very low risk for *Escherichia coli* O157:H7. In this study, Sorbitol-MacConkey agar did not work well on the isolation of *Escherichia coli* O157:H7 in catfish and showed a low sensitivity of detection because many

Pseudomonas spp. and some slow sorbitol-fermenting *Escherichia coli* affected the result of detection of *Escherichia coli* O157:H7. *Pseudomonas* spp., a kind of sorbitol fermentation-negative bacteria like *Escherichia coli* O157:H7 abundantly exist in seafood. However, only 50% of *Pseudomonas* spp. can be inhibited by the antibiotic, cefsulodin, which is added in the enrichment broth (Harold, 1984). In other words, the isolation agar was difficult to use for isolating and identifying *Escherichia coli* O157:H7 and *Pseudomonas* spp. Following the standard methods, conventional plating-culture methods and PCR based methods, sensitivity of Sorbitol-MacConkey agar was only 22% when 1 cfu *Escherichia coli* O157:H7 per gram catfish fillet was incubated in modified Tryptone Soya Broth with V.C.C supplement. Meanwhile, the study showed antibiotics, V.C.C supplement in the enrichment broth, also decreased the sensitivity of detection for *Escherichia coli* O157:H7 from 80% to 35% since the growth of *Escherichia coli* O157:H7 in the Modified Tryptone Soya Broth was also significantly inhibited by V.C.C supplement when the initial number of *Escherichia coli* O157:H7 is low.

Another factor in the detection of *Escherichia coli* O157:H7 in catfish is the order of PCR confirmation and isolation based on USDA and FDA methods. Multiplex PCR without isolation can reach 100% sensitivity of detection for *Escherichia coli* O157:H7 on catfish in Modified Tryptone Soya Broth without antibiotics. Regardless of whether or not antibiotics were included in enrichment broth multiplex PCR without isolation on detection of *Escherichia coli* O157:H7 in catfish showed a higher sensitivity than multiplex PCR with Sorbitol MacConkey agar, but similar sensitivity to multiplex PCR with Rainbow® Agar O157. If Sorbitol MacConkey agar was needed before multiplex PCR, the number of colonies chosen and tested by PCR should be increased.

Currently, there are numerous modified methods to improve isolation agar of *Escherichia coli* O157:H7 using chromogenic technology. The study showed that4-methylumbelliferyl-bata-D-galactopyranoside (D2) can be used to decrease the effect from false positive bacteria on Sorbitol-MacConkey agar, like *Pseudomonas* spp. because it also utilizes the β-galactosidase activity of *Escherichia coli* with the exception of the sorbitol negative fermentation characteristic (Fujisawa, 2000). Rainbow® Agar O157 is a commercial agar with chromogenic enzyme substrates that are associated with the β-galactosidase and β-glucuronidase (Anonymous, 2003). The sensitivity (100%) can be yielded when no antibiotics are in the enrichment broth, but still yielded a low sensitivity, 44%, when antibiotics were added to the enrichment broth. Under these circumstances, Rainbow® Agar O157 is able to compete with direct PCR on detection of *Escherichia coli* O157:H7 on catfish, but the price of the chromogenic agar is expensive.

Every food product possesses specific characteristics, so some improvements in detection of *Escherichia coli* O157:H7 in different kinds of foodstuff need to be accomplished by optimizing the detection procedures and media based on background microorganisms and the property of *Escherichia coli* O157:H7 in foodstuffs. Seafood has a large number of background microorganisms. In catfish, the number of *Pseudomonas* spp. is far more than the number of *Escherichia coli* O157:H7 and they possess similar biochemical characteristics, such as non-sorbitol fermentation. Thus, method development can reduce the false detection on the low-risk food for *Escherichia coli* O157:H7 and increase the efficiency of detection.

Table 1. Incidence of *Escherichia coli* O157:H7 and Identification of False Positive in Live Catfish, Pond Water and Sediments (Summer/Fall in 2010)

Source	Season	N_0[1]	N_1[2]	N_1[3]	False Positive[4]
Live Catfish	Summer	120	80	0	*Escherichia coli*/ *Pseudomonas* spp.
	Fall	210	5	0	*Escherichia coli*/ *Pseudomonas* spp.
Pond Water	Summer	20	10	0	*Escherichia coli*/ *Pseudomonas* spp.
	Fall	35	1	0	*Escherichia coli*/ *Pseudomonas* spp.
Pond Sediment	Summer	20	11	0	*Escherichia coli*/ *Pseudomonas* spp.
	Fall	35	1	0	*Escherichia coli*/ *Pseudomonas* spp.

[1] the total number of sample
[2]the incidence number of *Escherichia coli*
[3] the incidence number of *Escherichia coli* O157:H7 detected
[4] presumptive colorless colonies were not *Escherichia coli* O157:H7based on detected PCR, and then were identified by API20E

Table 2. The Relationship between the Number of Viable *Escherichia coli* O157:H7 Cells and Optical Density

The Number of Viable Cells (cfu/mL)	Optical Density
$<10^5$	0.001-0.009
10^5-10^7	0.010-0.099
10^7-10^9	0.100-0.999

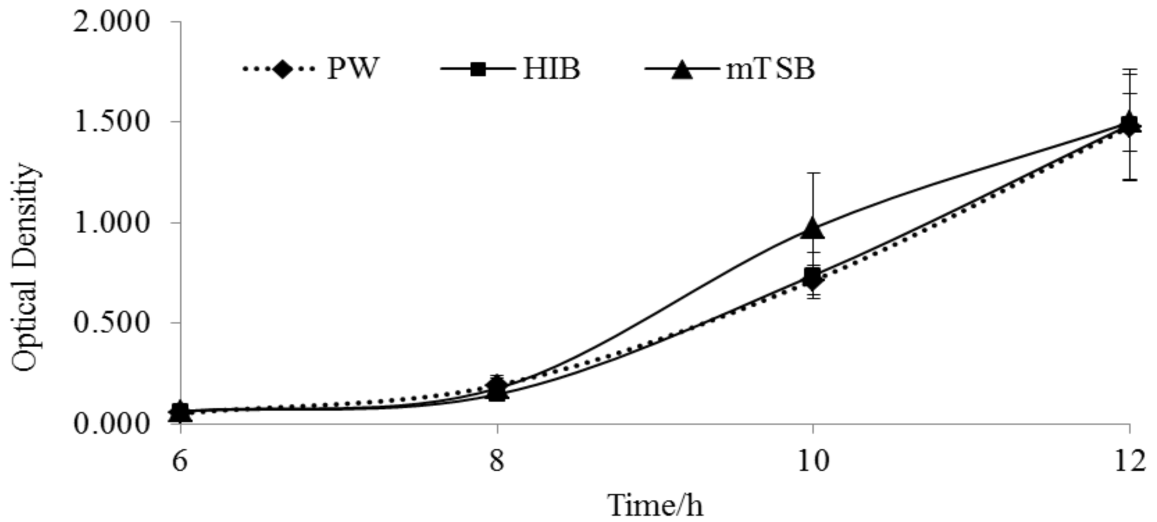

Figure 1 Growth Curve of *Escherichia coli* O157:H7 as Influenced by Enrichment Media Types[2]

[1]Standard Deviation was labeled in the figure.
[2]PW: Buffered Peptone Water
HIB: Brain Heart Infusion Broth
mTSB: Modified Tryptone Soya Broth

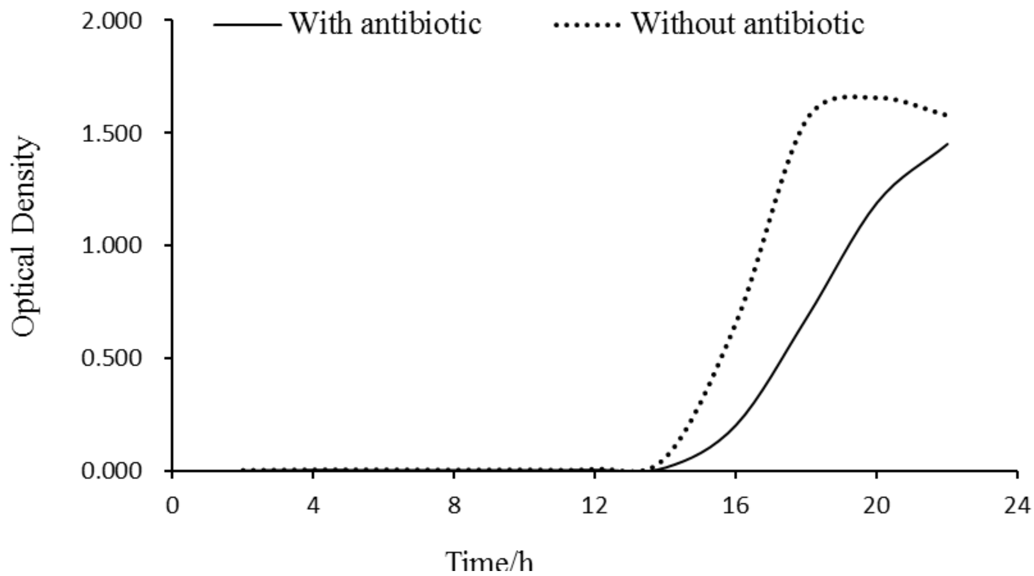

Figure 2 Growth Curve of *Escherichia coli* O157:H7 as Influenced by Antibiotics[1] in Modified Tryptone Soya Broth

[1]antibiotic: V.C.C Supplement containing vancomycin, cefsulodin and cefixime

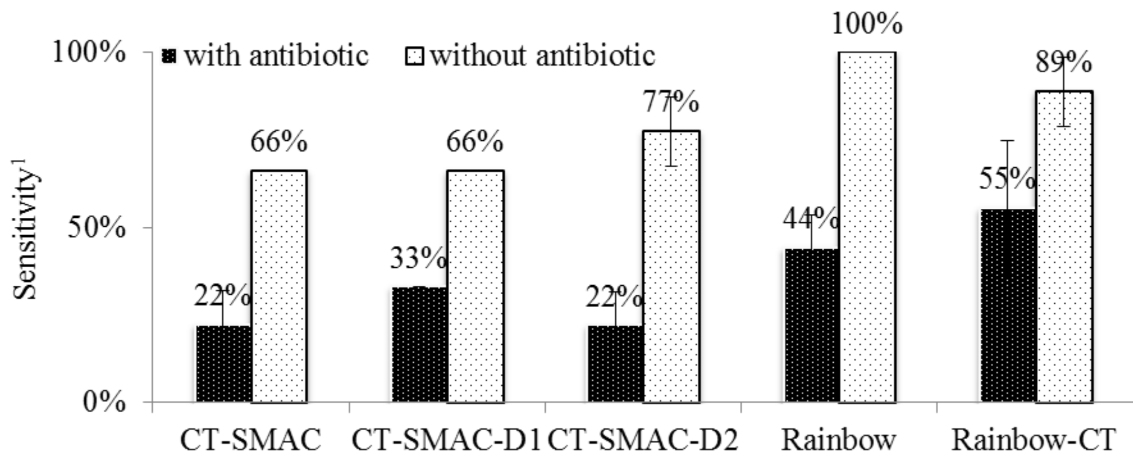

Figure 3 Sensitivity of Isolation Agar[2] for *Escherichia coli* O157:H7 in Catfish after Enrichment with/without Antibiotic[3]

[1]Sensitivity is the percentage of the number of true positives in the number of inoculation tests. Mean values and standard deviations were labeled in the figure.
[2]Isolation agar:
CT-SMAC: Sorbitol MacConkey Agar with cefixime and tellurite
CT-SMAC-D1: CT-SMAC with 4-Methylumbelliferyl-beta-D-ylopyranoside
CT-SMAC-D2: CT-SMAC with 4-Methylumbelliferyl-bata-D-galactopyranoside
Rainbow: Rainbow®Agar O157
Rainbow-CT: Rainbow®Agar O157 with cefixime and tellurite
[3]antibiotic: V.C.C Supplement containing vancomycin, cefsulodin and cefixime

CHAPTER IV

DEVELOPMENT OF A RAPID DETECTION METHOD FOR *ESCHERICHIA* O157:H7

Abstract

A rapid and sensitive detection method for *Escherichia coli* O157:H7 can help prevent against the more severe outbreaks and spreads of pathogens and the transmission of *Escherichia coli* O157:H7among food sources. The objective of this study was to develop a rapid, economical and convenient detection method for *Escherichia coli* O157:H7 in foods. The combination between pH Indicator I with Chemical 1 in the bottom color agar distinguished *Escherichia coli* O157:H7 from other bacteria by visual color on the bottom of the detection tube. Ground beef was chosen to optimize the detection tube and achieve the optimal ingredients for detection: the bottom color agar; Broth 2B with 1.5% agar, Antibiotic An, Chemical 1 and Chemical 2; and Agar B formed three layers in the detection tube, and then Broth M was chosen as the enrichment broth. The optimal incubation temperature was confirmed to be 42℃. The sensitivity, specificity and accuracy of the 24h rapid detection tube were 99.6%, 96.2% and 97.0% with an original inoculation of 10-100 cfu/g food matrixes. This rapid detection method can be used to detect *Escherichia coli* O157:H7 on food products and other samples rapidly, economically and conveniently.

Introduction

Escherichia coli O157:H7 has been recognized as a major public health pathogen because of low infectious dose but high health risk (Buchanan, 1997). A rapid and sensitive detection method for *Escherichia coli* O157:H7 can help prevent against the more severe outbreaks and spreads of pathogens and the transmission of *Escherichia coli* O157:H7 among food sources (Gould, 2005). Conventional plating-culture methods, biochemical methods, antibody-antigen immunological detection, PCR based detection, biosensors and GFP fluorescence were the main detection and identification methods for *Escherichia coli* O157:H7 (Deisingh, 2004).

In recent years, several rapid detection methods have been developed for testing *Escherichia coli* O157:H7. PCR and immunological detection methods are utilized widely because of high sensitivity, but high detection limits and time and labor costs inhibit the common use of the methods. Conventional plating-culture possesses the advantage of low detection limits and ease for handling, so they are still utilized by USDA and FDA as preliminary isolation or confirmation tests after PCR based detection. Some tests for *Escherichia coli* O157:H7 only utilize solid plating media and are highly reliable, but actually, most of them mainly make use of chromogenic enzyme substrates associated with β-galactosidase and β-glucuronidase to differentiate *Escherichia coli* O157:H7 from other bacteria. Restaino (2003) patented an easy and rapid method based on plating media that also combine chromogenic substrate and pH indicator dye to present different colors and indicate the presence of *Escherichia coli* O157:H7. However, no study has focused on only using biochemical tests of plating media and simple bacterial characteristics to rapidly detect *Escherichia coli* O157:H7 so that economical and convenient.

The objective of this study was to develop a rapid, economical and convenient detection method for *Escherichia coli* O157:H7 and apply and evaluate it in true food matrixes that have been associated with *Escherichia coli* O157:H7 contaminations.

Materials and Methods

Bacterial Strains

Four *Escherichia coli* O157:H7 Strains, ATCC43890, ATCC43895, ATCC35150 and one strain from USDA, were evaluated in inoculation tests. Generic *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC 27853 were used in this study as false-positive contaminants. A bacterium isolated from lettuce as false positive for *Escherichia coli* O157:H7 on the Sorbitol-MacConkey agar with antibiotic and identified by API20E (BioMerieu, La Palme) as *Enterobacter applomerans* was also used in the study. All strains were maintained at -20 ℃ in Trypticase Soy Broth (TSB) (Becton, Dickinson and Company).

Culture Media and Chemicals

Trypticase Soy Broth (Becton, Dickinson and Company), Broth M (Oxoid Ltd., Basingstoke), Broth 2B (Oxoid Ltd., Basingstoke) and Broth 1B (Oxoid Ltd., Basingstoke) were used as enrichment broths in this study. In addition, Agar B (Becton, Dickinson and Company) and Sorbitol-MacConkey Agar (Becton, Dickinson and Company) were prepared. Antibiotic An was purchased from Oxoid Ltd., Basingstoke and Agar Granulated (Becton, Dickinson and Company) were also used.

Sodium Chloride (Fisher Science Education), Chemical 2 (Fisher Science Education), Malachite Green Oxalate (Acros Organics, Thermo Fisher Scientific), 98% D-Sorbitol (Acros Organics, Thermo Fisher Scientific), Chemical 1 (National Aniline

Division), pH indicator III (Sigma Chemical), pH indicator II (Acros Organics, Thermo Fisher Scientific) and pH indicator I (Amresco, Solon Ind.) were used to prepare media.

Inoculated Food

Three brands of ground beef (80% lean) and three brands of iceberg lettuce were purchased from local supermarkets (Starkville, MS) and then inoculated with *Escherichia coli* O157:H7.

Development of Agar in Detection Tube for *Escherichia coli* O157:H7

Based on the ingredients of Sorbitol MacConkey agar, pH indicator I, II, III were evaluated as pH indicators in the bottom of the detection tube. The Chemical 1 was also added to improve the color distinction. The efficiency of the detection tube using different pH indicators were compared by visual color. The Antibiotic An was also added to test the effect of color in the detection tube. A high concentration of sorbitol, 2%, was also compared with 1.5% in the commercial Sorbitol MacConkey agar in the detection tube. After 24h incubation, the sensitivities were tested by the color at the bottom of the tube. Based on results, the optimal agar ingredients were achieved.

Development of Enrichment Media in Detection Tube on *Escherichia coli* O157:H7

The three enrichment media evaluated were Broth M (M), Broth 1B (1B) and Broth 2B (2B). All the enrichment media containing *Escherichia coli* O157:H7 were added in the detection tube and then incubated at 37℃for 24 h. Results were identified by the color of the bottom agar in the tube using a RGB label (R means red, G means green and B means blue). When the color of the bottom agar was in the range between R=255, G=127, B=0 and R=255, G=193, B= 37, the detection tube was reported as positive. If the color was out of the range, the result was reported as negative. In addition, the color

was also digitized with a Hunter Lab spectrocolorimeter (Reston, VA). Hue value was calculated by a-value and b-value ($a>0$ means red and $a<0$ means green; $b>0$ means yellow and $b<0$ means blue) by the formulation as below:

Hue= arctan (b/a), $a>0$, $b>0$;

Hue= 360 °+ arctan (b/a), $a>0$, $b<0$;

Hue= 180 °+ arctan (-b/a), $a<0$, $b>0$.

If the hue value was between 20 °and 160 °, the tube was identified as positive *Escherichia coli* O157:H7 test and then PCR was followed to confirm the presumptive culture tube. The sensitivity was then calculated using the below formula:

Sensitivity %= number of true positives/ number of true inoculated tests.

Correlation Middle Agar and Enrichment Media in Detection Tube on *Escherichia coli* O157:H7

Two enrichment media chosen, Broth M(M) and Broth 2B (2B) were combined with a middle agar layer, Broth 2B with 1.5% agar (A2B) or Broth M with 1.5% agar (AM). Four kinds of detection tubes were incubated at 37°C for 24 h after inoculation. Results were identified by the color of the bottom of the tube and measured with a Hunter Lab spectrocolorimeter (Reston, VA). Hue value and sensitivity were calculated using the formulas listed above.

Effect of Temperatures on Detection of *Escherichia coli* O157:H7

Pseudomonas aeruginosa was isolated and identified as false positive bacteria on the Sorbitol MacConkey Agar in the survey of *Escherichia coli* O157:H7 in catfish (previous experiment). A false positive bacterium that cannot ferment sorbitol and isolated from lettuce was identified as *Enterobacter applomerans* by API20E. These two

kinds of bacteria were utilized to evaluate the effect of temperature in the enrichment and isolation for detection of *Escherichia coli* O157:H7.

Effect of Temperatures on Growth of Escherichia coli O157:H7, Pseudomonas aeruginosa and Enterobacter applomerans

The effect of temperature on the growth of *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* and *Enterobacter applomerans* were measured separately by testing the number of viable *Escherichia coli* O157:H7 cells formed in Broth M at different incubation times. Enrichment broth tubes containing *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* and *Enterobacter applomerans* cells (less than 10 cfu/mL) separately, were incubated at 37℃ and 42℃, and enrichment cultures were collected at 6h, 8h, 10h, 12h and 14h. The number of viable *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* and *Enterobacter applomerans* cells formed in the enrichment broth tube were estimated by the optical density (O.D.) which was measured using a spectrophotometer (UV-1201, Shimadzu) at 600nm.

Effect of Temperatures of Enrichment and Isolation on Detection of Escherichia coli O157:H7

The enrichment broth tube containing *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* and *Enterobacter applomerans* cells (less than 10cfu/mL) were incubated at 37℃ and 42℃. After 24h enrichment, the bacteria cultures were diluted to 100cfu/mL, and then planted on Sorbitol MacConkey agar with antibiotic. Two agar incubation temperatures, 37℃ and 42℃, were evaluated. All the colorless colonies were identified by PCR. The sensitivity was calculated as the number of true positive colonies by the number of total colonies. Experiments were conducted on five replications, and the sensitivity of four different combination of temperature was compared.

Effect of Ratios of Escherichia coli O157:H7, Enterobacter applomerans, Pseudomonas aeruginosa under Different Temperatures of Enrichment and Isolation on Detection of Escherichia coli O157:H7 in Lettuce

Lettuce was chosen as the substrate to evaluate the effect of inoculum mixture, 1:1:1, 1:10:10, 1:50:50 and 1:100:100 of *Escherichia coli* O157:H7: *Enterobacter applomerans*: *Pseudomonas aeruginosa,* at different temperatures of enrichment and isolation on sensitivity of detection for *Escherichia coli* O157:H7. Inoculation concentration of *Escherichia coli* O157:H7 was 10-100 cfu/g lettuce. Other bacteria were mixed at the respective ratios. Two enrichment temperatures, 37°C and 42°C and two agar incubation temperatures, 37°C and 42°C were combined to evaluate the sensitivity of the tube.

Effect of Temperatures on Detection of Escherichia coli O157:H7 in Lettuce

The ratio of 1:10:100 (*Escherichia coli* O157:H7: *Enterobacter applomerans*: *Pseudomonas aeruginosa*) was chosen to evaluate the effect of temperatures of enrichment and isolation on detection of *Escherichia coli* O157:H7 in lettuce. After 24 h enrichment at 37°Cor 42°C, 100 μL of approximately 100 cfu/mL enrichment broth were plated on Sorbitol MacConkey agar at 37°Cor 42°C 24 h. Five replications were tested, and the sensitivities of detection at different enrichment and isolation temperatures were compared.

Optimization of Detection Tube for *Escherichia coli* O157:H7 in Food Matrixes

Ground beef (80% lean) was chosen as a food matrix to optimize the detection tube by inoculating 10-100 cfu/g of *Escherichia coli* O157:H7 in the ground beef. A number of samples were not inoculated to serve as negative controls. Ten different combination treatments were tested, and results were recorded with the Hunter Lab

spectrocolorimeter. Based on the color results (Hue value) and PCR confirmation, the total number of false positives and false negatives were recorded to calculate the sensitivity, specificity and accuracy using the formulas below (Eijkelkamp 2009):

Accuracy %= (number of true positives and true negatives)/ number of tests

Sensitivity %= number of true positives/ number of true inoculated tests

Specificity%= number of true negatives/ number of true non-inoculated tests

Evaluation of Detection Tube on *Escherichia coli* O157:H7

Three brands of 80% lean ground beef and three brands of iceberg lettuce were chosen as food matrixes to evaluate the detection tube. On each food sample, four strains of *Escherichia coli* O157:H7 (10-100 cfu/g) were inoculated to test the detection tube. Ten tubes replication was conducted for each strain inoculation test and ten tubes for non-inoculation test. Based on positive detection tube of ten inoculation tubes for each strain, sensitivity of the detection tube was calculated using the formulas listed above. Based on negative detection tube of ten non-inoculation tubes, specificity of detection tube was calculated using the formulas listed above. Meanwhile, accuracy of detection tube was also calculated.

Statistical analysis

A split plot design with a randomized complete block whole plot was used to evaluate sensitivity of detection for four strains of *Escherichia coli* O157:H7 under two types of foodstuffs (ground beef and iceberg lettuces). For each foodstuff, three supermarket products were chosen as replications (block). On each of two foodstuffs (whole plot), four strains of *Escherichia coli* O157:H7 (subplots) were inoculated separately. The sensitivities of detection were calculated and the results were analyzed

using PROC GLM in SAS (SAS 9.2, SAS Institute Inc.). When significant differences ($P<0.05$) occurred among different strains and different foodstuffs existed, the Fisher's least significant difference test was used to separate treatment means.

For specificity of detection, a randomized complete block design was also used to research the effect of foodstuff types on the specificity of the detection tube, and three supermarket products for each foodstuff were chosen as replications (block).The specificity of the detection tube was calculated, and then results were analyzed using PROC GLM in SAS (SAS 9.2, SAS Institute Inc.). When significant differences ($P<0.05$) occurred among different foodstuffs, the Fisher's least significant difference test was used to separate treatment means.

Results

Development of Agar and Enrichment Media in Rapid Detection Tube on *Escherichia coli* O157:H7

The choice of pH indicator in the media is important because the shift in pH changes the color of the media. Table 3 showed the different color of media by different pH indicators when different bacteria grow. The pH indicator II or pH indicator III was shown the visual color of generic *Escherichia coli* and *Escherichia coli* O157:H7 on the bottom of the detection tube were similar, but pH indicator I can distinguish generic *Escherichia coli*, *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* with pink, yellow and purple red. Growth of generic *Escherichia coli* can decrease pH because it can ferment sorbitol to acetic acid (Evryll, 1980). *Escherichia coli* O157:H7 are negative sorbitol-fermenting bacteria. They have to break protein down to ammonia in an aerobic environment to support their growth in the Sorbitol MacConkey agar (March, 1986). The

pH value was increased to more than 8.0. However, the deamination reaction cannot process without oxygen, so *Escherichia coli* O157:H7 needs to utilize an activity of decarboxylase to support their growth, and then the medium will be weakly basic (pH is 8.0). In addition, Chemical 1 also affects the final color. Under alkaline conditions, the solution of Chemical 1 will be decolorized because colorless product C1' is produced. At neutral pH, Chemical 1 solution will be violet, and at weak acid, it will be green violet. The combination of Chemical 1 and pH indicator I can improve in color distinction between pink (Hue = 354°) and yellow (94°) because the shift in pH is in the range between 6.8 and 8.0. In addition, pure *Pseudomonas aeruginosa* cannot grow in the agar of the tube due to its anaerobic characteristic, so the agar in the detection tube will retain its original neutral color, reddish purple (Hue =356°). The pH indicator I with chemical 1 is a better choice for the *Escherichia coli* O157:H7 detection tube.

Besides pH indicators, additional sorbitol cannot improve the distinction of color reaction in the detection tube. Increasing sorbitol from 1.5% to 2.0% made generic *Escherichia coli* react to a light pink, and then made a distinction between false and true positives less obvious (Table 4). The addition of Antibiotic An also resulted in less distinction of color reaction between *Escherichia coli* O157:H7 and the other bacteria that were tested (Table 5). Thus, pH indicator I with Chemical 1, 1.5% sorbitol without Antibiotic An are the best mix to differentiate bacterial cells that were present in the tube.

Sensitivity is defined as the percentage of the number of true positives to the total number of inoculation tests (Eijkelkamp, 2009). True positive tests mean the color of the agar was declared as positive and the enrichment culture was confirmed by PCR. In Table 6, M, 2B and 1B as enrichment broth in the detection tube were evaluated, but there is no difference among them ($P=0.3786>0.05$). Although M provided more

nutrients (carbohydrate and protein) to support the growth of *Escherichia coli* O157:H7, acid that is produced in the M can migrate into the bottom agar and produce a pink color that cannot be differentiated from the pink color of generic *Escherichia coli* in the tube.

Correlative Effect of Middle Agar and Enrichment Media in Detection Tube on *Escherichia coli* O157:H7

In order to increase the sensitivity of the detection tube, a middle agar layer was added to prevent enrichment broth from migrating into the isolation agar layer. There is no interaction between the combinations of enrichment media and middle agar and the sensitivity of detection for different strains of *Escherichia coli* O157:H7 (P=0.5592>0.05) and no difference among different strains of *Escherichia coli* O157:H7 (P=0.6017>0.05) (Table 7). Although there is no difference among different combinations of enrichment media and middle agar (P=0.6333>0.05), M with 1.5% agar (AM) probably breaks into the bottom agar, and then reduced the sensitivity of detection for *Escherichia coli* O157:H7 due to gas that was produced by carbohydrate fermentation (Table 8).

Effect of Temperature on Detection for *Escherichia coli* O157:H7

Escherichia coli O157:H7, *Enterobacter applomerans* and *Pseudomonas aeruginosa* showed colorless colonies on Sorbitol-MacConkey Agar because they cannot ferment sorbitol. Thus, *Enterobacter applomerans* and *Pseudomonas aeruginosa* affect the sensitivity of Sorbitol-MacConkey Agar. According to the growth curves for the three bacteria at different temperatures, 42°C enrichment can stimulate growth of *Escherichia coli* O157:H7 and inhibit *Enterobacter applomerans* growth more than 37°Cenrichment, but it could not prevent the growth of *Pseudomonas aeruginosa* (Figure 5). When comparing the four different combinations (enrichment and isolation) of pure *Escherichia*

coli O157:H7, both 42°C enrichment and 37°C isolation combination (USDA method) and 37°C enrichment and 37°C isolation combination (FDA method) possessed low detection sensitivities (65% and 68%). However, false positive ratios were reduced, and then sensitivities were increased from 65% and 68% to 100% and 94%, respectively for 42°C isolation instead of 37°C (Table 9). Thus, increasing both enrichment and isolation temperatures to 42°Ccan inhibit false positive bacteria and then increase sensitivity.

Because food matrix conditions are more complex than pure broth on detection of *Escherichia coli* O157:H7, lettuce was chosen to test the effect of temperature on detection. The number of false positive bacteria was also increased in the mixture ratio. When the number of false positive bacteria was added at the ratio of 1:1:1 (*Escherichia coli* O157:H7: *Enterobacter applomerans*: *Pseudomonas aeruginosa*), no false positive colony was detected on Sorbitol MacConkey agar at four temperature combinations (Table 10). When the initial numbers of false positive bacteria were increased, the ratio was changed to 1:10:10, only 37°C enrichment and 37°C isolation resulted in the growth of false positive bacteria. When the ratios were changed to 1:100:100, sensitivities were decreased at both the 42°C enrichment and the 37°C isolation combination and the 42°C enrichment and the 42°C isolation combination. By researching false positive bacteria under four temperature combinations, 42°C isolation probably improved the sensitivity of detection by inhibiting the growth of *Pseudomonas aeruginosa* completely. 42°C enrichment probably increased the sensitivity of detection by inhibiting the growth of most of *Enterobacter applomerans*. Figure 5demonstrated that part of *Enterobacter applomerans* still can slowly grow during the enrichment at 42°C. For a higher sensitivity of detection under a large number of *Enterobacter applomerans,* 37°C enrichment and 42°C

isolation should be considered to replace 37℃ enrichment and 37℃ isolation (FDA method) or 42℃ enrichment and 42℃ isolation (USDA method).

When *Pseudomonas aeruginosa* were the main false positive bacteria (1:10:100 was chosen), the sensitivity of detection was similar and high (>99%) when enrichment temperature was 42℃ regardless of isolation temperature. At 37℃ enrichment temperature, sensitivity was higher at 42℃ than at 37℃ isolation (Table 11).

Optimization of Detection Tube for *Escherichia coli* O157:H7 in Food Matrixes

Ground beef was chosen as the food matrix to optimize detection tube because of high incidence of *Escherichia coli* O157:H7 (CDC, 2011).In Table 13, for increasing the reliability (sensitivity, specificity and accuracy), Antibiotic I (An) was added in middle agar (A2B). Sensitivity, specificity and accuracy were increased to 55%, 55% and 55%. Although sensitivity was improved by adding Malachite Green oxalate (G) in middle agar, the low specificity could not be accepted. Agar B and 42℃ incubation temperature were chosen to improve specificity of detection. Chemical 1 (C1) and Chemical 2 (C2) were also added to improve sensitivity, specificity and accuracy to 80%, 90% and 90% by inhibiting the growth of gram positive bacteria. The optimal ingredients of detection tube were confirmed as below:

Three layers isolation agar: the bottom color isolation agar; Broth 2B with 1.5% agar, Antibiotic An, Chemical 1 and Chemical 2; and Agar B.

Enrichment broth: Broth M.

Incubation condition: 24h at 42℃.

Evaluation of Optimal Detection Tube for *Escherichia coli* O157:H7

There was no difference among different types of foodstuffs (ground beef and iceberg lettuce) ($P=0.1427>0.05$). And the sensitivities of detection tube were similar for different strains of *Escherichia coli* O157:H7 ($P=0.1884>0.05$), and there is no interaction between different strains and food types ($P=0.3280>0.05$). On average, based on total 659 tests, the sensitivity of detection tube was 96.6%.

For specificity of detection tube, there is no difference between ground beef and iceberg lettuce ($P=0.4226>0.05$). On average of 149 tests, the specificity of detection tube was 99.2%. In addition, totally, the accuracy of detection tube was 97.0%.

Discussion

Conventional plating is the main detection method for *Escherichia coli* O157:H7 since it allows for low detection limits and labor/time costs. Many biochemical materials, such as chromogenic substrates or antibiotics have been added to the conventional plating culture method and produce easier and more rapid test kits (Anonymous, 2003; Restaino, 2003), but they also reduced the benefits of the traditional conventional plating culture methods, low cost and low detection limits. In this study, a rapid, economical and convenient detection method was developed by bacterial biochemical characteristics. Temperature is an essential factor for bacterial growth, but its importance is often overlooked. This study indicated that different temperatures of enrichment and isolation combinations showed the sensitivity of conventional plating detection method for *Escherichia coli* O157:H7. The isolation temperature 42°C can inhibit the growth of *Pseudomonas aeruginosa* but not 37°C, whereas 42°C enrichment temperature can slow the growth of *Enterobacter applomerans* down but not 37°C. After comparing four temperature combinations in iceberg lettuce, the 37°C enrichment and 42°C isolation

combination improved the sensitivity of Sorbitol MacConkey agar when *Enterobacter applomerans* were the main false positive bacteria. When *Pseudomonas aeruginosa* were the main false positive bacteria, 42℃ enrichment temperature regardless of isolation temperature or 37℃ enrichment and 42℃ isolation were chosen. When utilizing the detection tube, the 42℃ incubation temperature was also preferred because it increased sensitivity, specificity and accuracy simultaneously.

In addition to the temperature, the study also utilized another basic factor, pH indicator I with the bacterial biochemical characteristics in traditional Sorbitol MacConkey Agar. In Sorbitol MacConkey agar, *Escherichia coli* O157:H7 shows up as colorless colonies by its negative-sorbitol fermentation and increases the pH value of whole agar by its deamination characteristic. Other *Enterobacteriaceae,* such as generic *Escherichia coli* or other pathogenic *Escherichia coli*, appear pink because they can ferment sorbitol to produce lactic acid and decrease pH. *Escherichia coli* are facultative anaerobic bacteria that use carbohydrates by anaerobic fermentation and aerobic respiration, but they start using proteins to supply themselves when there is a lack of carbohydrates present. Thus, in the detection tube, *Escherichia coli* O157:H7 does not ferment carbohydrate that is grown in the bottom agar, and therefor they utilize proteins for growth. They cannot break protein down to ammonia and increase pH significantly under anaerobic conditions, but they increase the pH through decarboxylation. On the other hand, other bacteria cannot carry through the decarboxylation reaction because they ferment sorbitol to lactic acid for growth. (GALE, 1944) Thus, with pH indicator I, the third color (yellow) from decarboxylation distinguishes *Escherichia coli* O157:H7 from other bacteria easily, thus making expensive chromogenic substrates unnecessary. Additionally, the combination of the temperature and tube can take the place of the

cefsulodin antibiotic that is only able to inhibit the growth of 50% of *Pseudomonas aeruginosa* (Harold, 1984) because *Pseudomonas aeruginosa* cannot grow at 42°Cunder anaerobic conditions. Moreover, cefsulodin partly inhibits the growth of *Escherichia coli* O157:H7, so the recovery of *Escherichia coli* O157:H7 can be improved without this antibiotic.

In the Table 14, conventional plating-culture, PCR based method and the detection tube were compared. Gilbert showed the satisfactory microbiological detection limitation for *Escherichia coli* O157:H7 should be in the range between 20 and 100cfu/g (Gilbert, 2000). The limitation of detection tube was less than 100cfu/g. All of detection methods for *Escherichia coli* O157:H7 require a 24h enrichment step. After enrichment, expensive techniques and equipment, like PCR, ELISA or biosensors were needed to achieve a high sensitivity of detection with the exception of conventional plating methods (Deisingh, 2004). Without other expensive equipment and high trained personnel, the 24h rapid detection tube for *Escherichia coli* O157:H7 can achieve 96.6% sensitivity, 99.2% specificity and 97.0% accuracy in foodstuffs. However, the sensitivity, specificity and accuracy of conventional 48h Sorbitol MacConkey agar plating detection for pure *Escherichia coli* O157:H7 were only 94.1%, 91.6% and 93.4% and 48h Rainbow® agar O157 detection for pure *Escherichia coli* O157:H7 can also only reach 91.1% sensitivity, 91.6% specificity and 91.3% accuracy (Manafi, 2001). Thus, the detection tube for *Escherichia coli* O157:H7 is a rapid, economical and convenient method and it can be applied efficiently in foodstuffs to detect *Escherichia coli* O157:H7.

Table 3. Color of the Bottom Agar as Influenced by pH Indicators for Generic *Escherichia coli*, *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa*

pH Indicator	Generic *Escherichia coli*	*Escherichia coli* O157:H7	*Pseudomonas aeruginosa*
pH indicator I	Pink[1] (255,51,204)	Orange Yellow (255,128,0)	Reddish Purple (150,46,76)
pH indicator II	Blue Green (17,147,97)	Green Blue (27,131,121)	Purple Blue (74,140,134)
pH indicator III	Green Orange (219,141,77)	Orange (255,151,51)	Purple Orange (241,101,52)

[1]The results were shown by visual color and RGB value (Red, Green and Blue) were followed.

Table 4. Color of the Bottom Agar as Influenced by Sorbitol Concentration for Generic *Escherichia coli*, *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa*

Sorbitol Concentration	Generic *Escherichia coli*	*Escherichia coli* O157:H7	*Pseudomonas aeruginosa*
2%	White Pink[1] (255,121,172)	Orange Yellow (255,128,0)	Reddish Purple (150,46,76)
1.5%	Pink (255,51,204)	Orange Yellow (255,128,0)	Reddish Purple (150,46,76)

[1]The results were shown by visual color and RGB value (Red, Green and Blue) were followed.

Table 5. Color of the Bottom Agar as Influenced by Antibiotic An for Generic *Escherichia coli*, *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa*

Antibiotic An	Generic *Escherichia coli*	*Escherichia coli* O157:H7	*Pseudomonas aeruginosa*
With	White Pink[1] (255,121,172)	Orange Yellow (255,128,0)	Reddish Purple (150,46,76)
Without	Pink (255,51,204)	Orange Yellow (255,128,0)	Reddish Purple (150,46,76)

[1]The results were shown by visual color and RGB value (Red, Green and Blue) were followed.

Table 6. Sensitivity of Detection for *Escherichia coli* O157:H7as Influenced by Enrichment Media

Enrichment Media (n[4]=50)	Sensitivity[2]
1B[1]	68.3%[a3]
2B	82.2%[a]
M	82.2%[a]

[1]1B: enrichment Broth 1B; 2B: enrichment Broth 2B; M: enrichment M
[2]Sensitivity is the percentage of the number of true positives in the number of inoculation tests
[3]Same letter means there is no statistical difference (P=0.3786>0.05)
[4]n means total tests number of each media, S.E. =1.1%

Table 7. Sensitivity[1] of Detection for *Escherichia coli* O157:H7 as Influenced by the Combinations[2] of Enrichment Media and Middle Agar (N[3]=30)

	A2B+M	A2B+2B	AM+M	AM+2B	AverageS[4]
43890	73.3%	86.7%	83.3%	70.0%	78.3%[a6]
43895	83.3%	63.3%	76.7%	66.7%	72.5%[a]
35150	80%	73.3%	60%	73.3%	71.7%[a]
USDA	76.7%	83.3%	76.7%	73.3%	77.5%[a]
AverageM[5]	78%[a7]	77%[a]	74%[a]	71%[a]	75.0%[8]

[1]Sensitivity is the percentage of the number of true positives in the number of inoculation tests
[2]The four combinations of enrichment media and middle agar are from Broth 2B with 1.5% Agar Granulated (A2B) or Broth M with 1.5% Agar Granulated (AM) and Broth 2B (2B) or Broth M (M)
[3]N: total number of test for each strain on each combination method, S.E. =0.6%
[4]AverageS: sensitivities of detections for four strains of *Escherichia coli* O157:H7
[5]AverageM: sensitivities of four combination methods
[6]Same letter means there is no difference among different combinations ($P=0.6333>0.05$)
[7]Same letter means there is no difference among different strains of *Escherichia coli* O157:H7 ($P=0.6017>0.05$)
[8]Overall mean

Table 8. Sensitivity[1] of Detection for *Escherichia coli* O157:H7 as Influenced by Enrichment and Isolation Temperatures (N[2]=5)

	37°C Enrichment[3]	42°C Enrichment
37°C Isolation[4]	68%	65%
42°C Isolation	94%	100%

[1]Sensitivity is the percentage of the number of true positive colonies in the number of all colorless colonies
[2]N means the number of replication, S.E. =6.0%
[3]Enrichment: *Escherichia coli* O157:H7 Enrichment Broth without antibiotic
[4]Isolation: Sorbitol-MacConkey Agar with cefixime and tellurite

Table 9. Sensitivity[1] of Detection for *Escherichia coli* O157:H7 in lettuce as Influenced by Bacterial Strain Ratio[2] (*Escherichia coli* O157:H7: *Enterobacter applomerans: Pseudomonas aeruginosa*) and Temperature (Enrichment and Isolation) (N[3]=1)

Ratio	37°CEnrichment/ 37°C Isolation	37°C Enrichment/ 42°C Isolation	42°C Enrichment / 37°C Isolation	42°C Enrichment/ 42°C Isolation
1:1:1	100%	100%	100%	100%
1:10:10	67%	100%	100%	100%
1:50:50	50%	100%	80%	71%
1:100:100	54%	100%	93%	64%

[1]Sensitivity is the percentage of the number of true positives in the number of inoculation tests
[2]Ratio: *Escherichia coli* O157:H7: *Enterobacter* applomerans: *Pseudomonas* aeruginosa
[3]N means the number of test

Table 10. Sensitivity[1] of Detection for *Escherichia coli* O157:H7 as Influenced by Enrichment and Isolation Temperature at the Ratio of 1:10:100[2] (N[3]=5)

	37°C Enrichment	42°C Enrichment
37°CIsolaton	76%[b4]	99%[a]
42°C Isolation	100%[a]	100%[a]

[1]Sensitivity is the percentage of the number of true positive colony in the number of all colorless colonies
[2]Ratio: *Escherichia coli* O157:H7: *Enterobacter* applomerans: *Pseudomonas* aeruginosa
[3]N means the number of replication, S.E. =2.8%
[4]Different letters mean there are statistical difference (P=0.0257<0.05)

Table 11. Sensitivity and Specificity of Detection Tube for *Escherichia coli* O157:H7 in Ground Beef and Iceberg Lettuce

	Strain[3]	Ground Beef	Iceberg Lettuce	Average
Inoculation	438903	95.0%	96.7%	95.8%[a5]
Sensitivity[1]	43895	90.0%	97.8%	93.8%[a]
(n[7]=659)	35150	97.8%	98.9%	98.3%[a]
	USDA	98.8%	97.7%	98.3%[a]
	Average	95.3%[a4]	97.8%[a]	
Non-inoculation specificity[2](n=149)		98.3%[a6]	100%[a]	

[1]Sensitivity = number of true positives/ number of true inoculated tests
[2]Specificity= number of true negatives/ number of true non-inoculated tests
[3]Strains of *Escherichia coli* O157:H7, ATCC43890, ATCC 43895, ATCC35150 and one strain from USDA
[4]Same letter means there is no different sensitivity between ground beef and iceberg lettuce (P=0.1427>0.05, S.E. =0.2%)
[5]Same letter means there is no different sensitivity among different strains of *Escherichia coli* O157:H7 (P=0.0.1884>0.05)
[6]Same letter means there is no different specificity between ground beef and iceberg lettuce (P=0.4226 >0.05, S.E. =0.2%)
[7]n: the total number of test

Table 12. Parameters (Accuracy, Sensitivity and Specificity) of Detection Tube for *Escherichia coli* O157:H7

	Mean[1]
Accuracy[3] (n[6]=659)	97.0%
Sensitivity[4] (n=149)	96.6%
Specificity[5] (n=808)	99.2%

[1]Means are the average reliabilities of detection for *Escherichia coli* O157:H7 since no difference between different types of foodstuffs
[3] Accuracy = (number of true positives and true negatives)/ number of tests (S.E. =0.2%)
[4]Sensitivity = number of true positives/ number of true inoculated tests (S.E. =0.2%)
[5]Specificity= number of true negatives/ number of true non-inoculated tests (S.E. =0.2%)
[7]n: the total test number

Table 13. Sensitivity, Specificity and Accuracy of Detection for *Escherichia coli* O157:H7 under Different Treatments in Ground Beef

Middle Agar[1]	Enrichment Media	Temperature	Sensitivity[10]	Specificity[11]	Accuracy[9]
A2B	2B	37℃	33%(10/30)	17% (5/30)	25% (15/60)
A2B	M	37℃	15% (3/20)	45% (9/20)	30% (12/40)
A2B/An[2]	M	37℃	55% (11/20)	55% (11/20)	55% (22/40)
A2B/An/5ppm[4]G[3]	M	37℃	60% (6/10)	50% (5/10)	55% (11/20)
A2B/An/10ppmG	M	37℃	80% (8/10)	50% (5/10)	65% (13/20)
A2B/An/20ppmG	M	37℃	80% (8/10)	20% (2/10)	50% (10/20)
A2B/An/20ppmG	M	42℃	90% (9/10)	30% (3/10)	60% (12/20)
A2B/An/20ppmG+Agar B[5]	M	42℃	30% (3/10)	70% (7/10)	50% (10/20)
A2B/An/10ppmG+Agar B	M	42℃	70% (7/10)	56% (5/9)	63% (12/19)
Agar B+[8]A2B/An/C1[6]/C2[7]	M	42℃	80% (8/10)	90% (9/10)	85% (17/20)

[1]Middle Agar: the middle agar layer, Broth 2B with 1.5% agar (A2B)
[2]An: Antibiotic An added inA2B
[3]G: Malachite Green oxalate
[4] the concentration of Malachite Green oxalate is 5, 10 or 20ppM
[5]+Agar B: under the middle agar layer but above the isolation agar layer
[6] C1: Chemical 1
[7] C2: Chemical 2
[8]Agar B+: above the middle agar layer
[9] Accuracy = (number of true positives and true negatives)/ number of tests
[10]Sensitivity = number of true positives/ number of true inoculated tests
[11]Specificity= number of true negatives/ number of true non-inoculated tests

Table 14. Comparisons of Conventional Plating-Culture, PCR Based Method and Detection Tube

	Plating-culture		Detection tube		PCR
	Rainbow	CT-SMAC			
Cost/test[1]	$4.27	$2.12	<$1.00[2]		>>$2.3
Less than detection limitation[3]	Yes		Yes		Yes
Enrichment step	Yes		Yes		Yes
Trained person	Yes		No		Yes
Expensive equipment	No		No		Yes
PCR Confirm	Yes[4]		No[5]	Yes	PCR
Specificity[7]	97.9% (f[10])	42.7% (f)	99.6% (f)	=PCR[6]	
Sensitivity[8]	91.1% (p[11])	94.1% (p)	96.2% (f)		
Accuracy[9]	91.3% (P)	93.4% (P)	97.0% (f)		
Time[12]	48h	48h	24h		24h

1Estimated Cost is based on Thermo Fisher Scientific Inc. (www. fishersci.com)
[2]Estimated retail cost of tube
[3]The satisfactory detection limitation is in the range between 20 and 100 cfu/g
[4]After Isolation on the plate, presumptive colonies need be confirmed by PCR
[5]Detection tube can be confirmed by PCR, but it is optional.
[6]If needed, PCR can be conducted directly from the top broth of detection tube
[7]Specificity= number of true negatives/ number of true non-inoculated tests
[8]Sensitivity = number of true positives/ number of true inoculated tests
[9]Accuracy = (number of true positives and true negatives)/ number of tests
[10]f means the methods were tested in the foodstuffs
[11]P means the methods were tested in the pure bacterial culture
[12]Time doesn't include post-confirmation test.

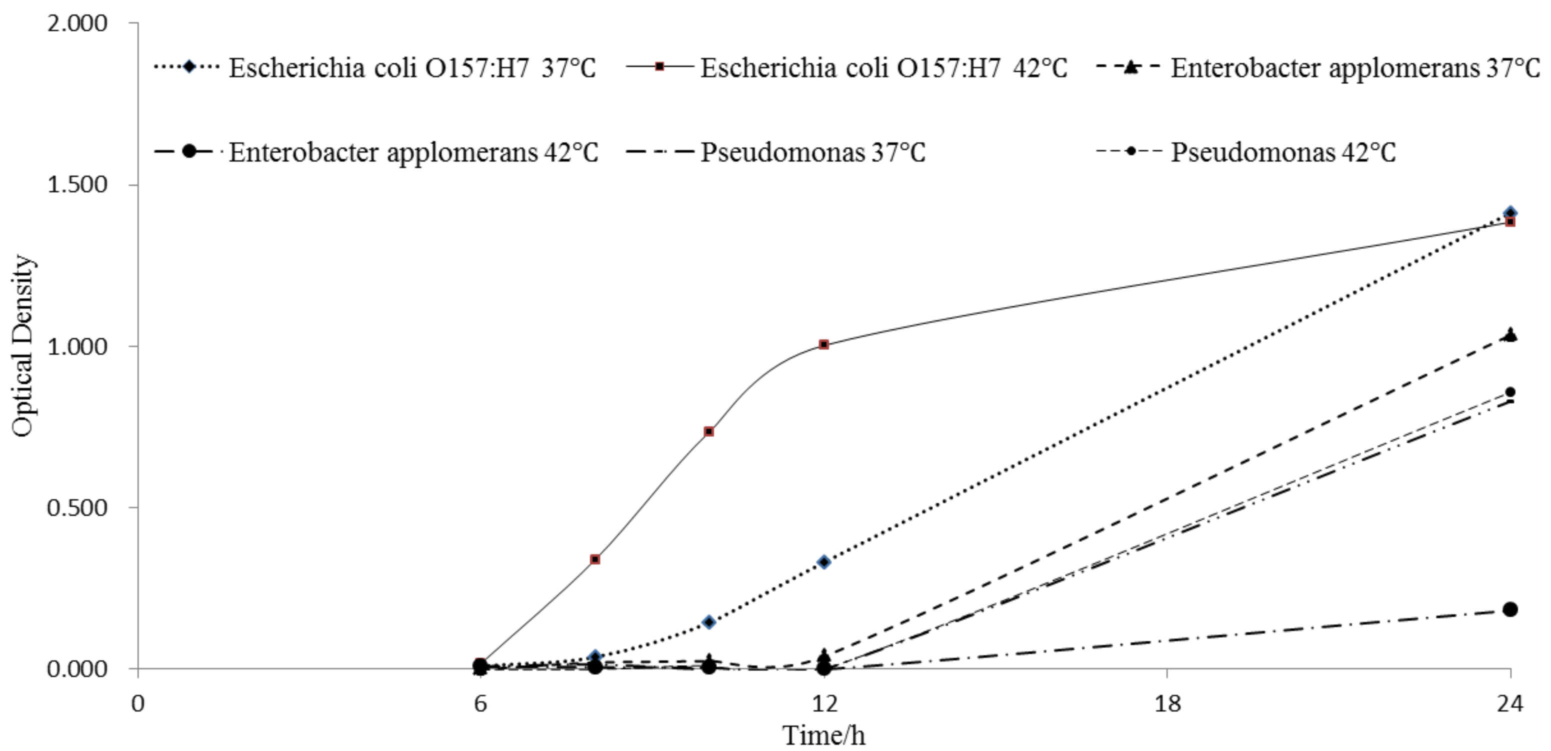

Figure 4. Growth Curves of *Escherichia coli* O157:H7,*Pseudomonas aeruginosa* and *Enterobacter applomerans*as Influenced by Different Enrichment Temperature

CHAPTER V

SUMMARY AND CONCLUSIONS

Using standard detection methods for *Escherichia coli* O157:H7 on Sorbitol-MacConkey agar, a high number of false positives was detected. This was the reason that other non-sorbitol fermenting bacteria, such as *Pseudomonas spp.*, generic *Escherichia coli* and *Enterobacter applomerans*, common in catfish during the warmer months, showed on the plates. Generic *Escherichia coli* ferment sorbitol slowly. *Pseudomonas* spp. cannot utilize sorbitol as energy like *Escherichia coli* O157:H7 and they can also grow on the Sorbitol MacConkey agar by deamination or decarboxylation.

Escherichia coli O157:H7 can grow in different enrichment media similarly. Antibiotics can improve the detection for *Escherichia coli* O157:H7 by inhibiting other bacteria, but they also inhibited the growth of *Escherichia coli* O157:H7 when the number of *Escherichia coli* O157:H7 was small. Additionally, a high number of false positives show on conventional plating method. By comparison, the sensitivity of Sorbitol-MacConkey agar was only 44% in the detection of *Escherichia coli* O157:H7 in catfish. Manafi (2001) also showed false positive rate of Sorbitol MacConkey agar was 53.7% for *Escherichia coli* O157:H7. Chromogenic substrates added cannot decrease the false positive ratio and the false negative ratio on Sorbitol MacConkey Agar. Even on some commercial chromogenic agars, the false positive ratios still were more than 2% when the number of *Escherichia coli* O157:H7 was high (Manafi, 2001). Besides,

Multiplex PCR without previous isolation for *Escherichia coli* O157:H7 possessed a high sensitivity, but high labor and time costs cannot be resolved.

Thus, a rapid, economical and convenient detection method was developed in this study. The pH indicator I with Chemical 1 was utilized to distinguish *Escherichia coli* O157:H7 with generic *Escherichia coli* and *Pseudomonas aeruginosa* by color. Under the starvation of carbohydrates and anaerobic condition, yellow color of the detection tube was produced by utilizing their decarboxylase activities. And false positive bacterium, *Pseudomonas aeruginosa* cannot grow in the bottom agar because of their anaerobic characteristics.

Temperature was another important factor for the sensitivity of detection for *Escherichia coli* O157:H7. Different enrichment and isolation temperatures affect the sensitivity of detection method for *Escherichia coli* O157:H7. The 37℃ enrichment and 42℃ isolation combination can minimize the effects of *Enterobacter applomerans* and *Pseudomonas aeruginosa* on Sorbitol MacConkey agar. When *Pseudomonas aeruginosa* was the main false positive, the sensitivity of detection was similar and high at 42℃enrichment temperature regardless of isolation temperature. The sensitivity of conventional plating method can be increased from 76% to more than 99%. The growths of false positive bacteria were inhibited, so 42℃ incubation temperature was also chosen to improve the sensitivity, specificity and accuracy of detection tube for *Escherichia coli* O157:H7.

Finally, the rapid detection method was optimized and evaluated in high risk foods: ground beef and iceberg lettuce. Agar B and buffered peptone agar with Chemical 1, Chemical 2 and Antibiotic An were combined to increase the sensitivity of the detection tube for *Escherichia coli* O157:H7 by inhibiting the growth of gram positive

bacteria. The reliability (sensitivity, specificity and accuracy) of the 24h rapid detection tube was similar for different types of foodstuffs. Under the 95% confidence level, 96.2% sensitivity, 99.6% specificity and 97.0% accuracy were achieved for the of detection tube. A more rapid and convenient 24h detection technology was developed in this study by comparing other conventional plating methods, immunological detection, PCR-based detection, biosensors for *Escherichia coli* O157:H7.

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