DETECTION OF SHIGA TOXIN-PRODUCING *Escherichia coli* AND *Salmonella* AEROSOLIZED IN VARIOUS AREAS OF COMMERCIAL SLAUGHTER PLANTS BY USING DYNAMIC BIOAEROSOL MONITORING TECHNIQUES

A Dissertation

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ZAHRA HASSAN MOHAMMAD

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Chair of Committee,	Alejandro Castillo
Committee Members,	Maria D. King
	Gary R. Acuff
	Davey Griffin
Head of Department,	Boon Chew

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ABSTRACT

Generation of bioaerosols containing bacterial pathogens during beef harvesting is an important issue to consider when controlling pathogens in the meat industry. Pathogens such as Shiga toxin-producing *Escherichia coli* (STEC) and *Salmonella* present on carcasses may be aerosolized during initial steps of slaughter, where highly active procedures are conducted, and transferred from these dirty areas to cleaner areas. This may result in the pathogens being deposited on the meat before the end product is released for sale. In this study, large air samples (2.8-4.8 m^3) were collected from various areas at two large and two small beef harvesting establishments during the fall spring and summer seasons using a wetted wall cyclone (WWC) sampler. Samples were tested for STEC and *Salmonella* and analyzed by direct plating, automated immunoassay system (Crystal Diagnostic, CDx), and real-time PCR (RT-PCR) analysis. All positive samples from CDx were confirmed by plating in selective and differential media followed by biochemical and serological tests, and STEC was confirmed by conventional PCR. Positive isolates were further confirmed using BAX PCR System. The RT-PCR products were confirmed by Illumina Sequencing. Based on selective plating, there were no positive air samples for *Salmonella* or STEC in the fall season, while air samples from all plants were positive for *Salmonella* and STEC in the spring and summer. The recovery of both pathogens was improved by extending enrichment time from 18 to 36 h. Percentages of positive samples when enriching for 18 h were 21.4 and 17.9 for *Salmonella* and STEC, respectively. When enriching for 36 h, percent positives were 57.1 and 60.7 for *Salmonella* and STEC, respectively. The percent positives when testing both *Salmonella* and STEC by RT-PCR (37.5%, 65.0%) were significantly higher than CDx with enrichment for 18 h ($P<0.05$). For 36 h enrichment,

Salmonella percent positives (57.1) by CDx were significantly higher than RT-PCR. While the percent of STEC positives was not different for either method (60.0%, 65.0%). PFGE analysis showed that the bacterial DNA from isolates from "dirty" areas was the same as the DNA from "clean" areas, indicating potential role of the air in transferring bacteria between different areas of the plant. In conclusion, the outcome of this study will help the meat industry be aware of the presence of bioaerosols in meat plants and the information will be used to enhance meat plants' sanitation processes and promote consumers' health.

DEDICATION

I dedicated this dissertation work to my husband, Dlzar Mohamed and my son Rawa Dlzar for their patience and love throughout this program.

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NOMENCLATURE

STEC	Shiga Toxin-Producing *Escherichia coli*
WWC	Wetted Wall Cyclone
h	Hour
min	Minute
CFU	Colony Forming Unit
ml	Milliliter
µl	Microliter
PFGE	Pulse Field Gel Electrophoresis
PBS	Phosphate Buffered Saline
RT-PCR	Real Time PCR
HUS	Hemolytic Uremic Syndrome
HC	Hemorrhagic Colitis
ELISA	Enzyme-Linked Immunosorbent Assay
MLG	Microbiology Laboratory Guidebook
FSIS	Food Safety and Inspection Service
BAM	Bacteriological Analytical Manual
CDC	Centers for Disease Control and prevention
UPEB	Universal Pre-Enrichment Broth
FSITL	Food Safety and Intervention Technology Research Lab

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CHAPTER I

INTRODUCTION

Contamination of meat and meat products by pathogenic and spoilage organisms is one of the major problems faced by the meat industry. Meat carcasses can be contaminated through several mechanisms and at various points during the processing of slaughtering and storage (Sofos et al., 1999a, Villarreal et al., 2016). One of the potential sources of contamination by pathogenic organisms is through the air (Kang and Frank, 1989). The presence of microorganisms in the air of meat harvesting establishments, with the consequences transfer of such microorganisms including potential pathogens to the carcasses, is a well-documented fact (Jericho et al., 2000; Okraszewska-Lasica et al., 2014; Pearce et al., 2006; Rahkio and Korkeala, 1997; Kotula and Emswiler-Rose, 1988; Burfoot et al., 2006; Schmidt et al., 2012). Among major sources of contamination in meat processing facilities, bioaerosols may be of great concern. These bioaerosols may be generated from wastewater, rinse water and spilled product (blood, feces, and the contents of animals' gut) that become aerosolized. Due to moisture commonly being high in the slaughter environment, heating, ventilation, and air conditioning (HVAC) systems can provide conditions for microbial growth and therefore contribute to airborne microorganisms under normal operation. Worker activity (including talking, sneezing, and coughing) may also generate bioaerosols. The correlation between worker activity and airborne bacteria has been reported by Heldman et al (1964). Equipment operation, sink and floor drain, and high pressure spraying can also be major generators of bioaerosols (Heldman et al., 1974, Salem and Gardner 1994).

Salmonella and Shiga toxin-producing *Escherichia coli* (STEC) are the greatest pathogens of concern in beef due to the prevalence of these microorganisms in the gastrointestinal tract of beef cattle (Beach et al., 2002; Davies et al., 2007; Swanenburg et al., 2001; Van Donkersgoed et al., 1999). Airborne transmission of bacterial pathogens such as *Salmonella* and STEC to the surface of beef cattle and pigs may occur at various steps during cattle raising and feeding (Proux et al., 2001). These pathogens may be transferred to the carcasses during harvesting by bioaerosols formed at specific processing areas such as hide removal in beef harvesting establishments, where air and droplets are more likely to be aerosolized (Pearce et al., 2006; Schmidt et al., 2012). In addition, these bioaerosols may transfer from unclean areas such as dehiding into clean areas such as chiller and fabrication rooms. *Salmonella* could survive and persist in aerosols for up to 4.5 hours, which indicates the potential for airborne dissemination (Plakhotya, 1984).

Air sampling at food plants helps to recognize the sources of airborne contamination with foodborne pathogens and allows the food industry to be aware of the potential risk of airborne organisms. Consequently, air sampling and testing can help to enhance their sanitation programs. Information on populations of total plate counts, lactic acid bacteria, yeasts, and molds etc. in the air of food and other types of premises has been available at least since the 1950's and 1960's (Kotula and Kinner, 1964; Cvjetanovic, 1958). However, previous studies on the prevalence of pathogenic bacteria such as *Salmonella*, *Listeria monocytogenes* or STEC have reported absence, low incidences, or low concentrations of the pathogens (Okraszewska-Lasica et al., 2014; Pearce et al., 2006; Schmidt et al., 2012; Sofos et al., 1999a). The results obtained by previous studies might underestimate the contamination present in the air because most of these reports have used plate sedimentation methods for air sampling. These methods typically involve the collection of

a relatively low volume of air resulting from sampling at a low air flow rate for a time ranging between minutes and few hours. Sampling for longer times to collect a larger air sample generally results in sample dehydration, which may cause stress to the bacteria. The low recovery of microorganisms in air samples collected by traditional bioaerosol sampling methods, such as spore traps, impingers, nucleopore and gelatin filters has been reported for several decades (Errington and Powell, 1969; Lin and Li, 1998). These methods may cause biological stress during the sampling process (Lin and Li, 1998), and the small volume of air sample that is collected by these methods, may result in underestimation of the prevalence of airborne organisms. In some of the previous studies, the air sampler was set at a flow rate of 28 L/ min or less, with collection sample times of 15 min (Jensen et al., 1992). Therefore in these studies, the volume of air that has been collected is less than 0.5 m^3. To overcome the old and current air samplers' problems, Wetted Wall Cyclone (WWC) air sampling methods were used in this study. The WWC has advantages over other collectors by continuously sampling large volumes of air for a longer time and collecting bioaerosols into a liquid solution with air flow rates of 100-300 L/ min (Hu and McFarland, 2007) that prevents cell stress due to sample drying. These samplers have been developed at the Aerosol Technology Laboratory at Texas A&M and are found to be highly effective at collecting bioaerosols (McFarland et al., 2010; King and McFarland, 2012) and have been used in almost real-time monitoring of microorganisms in air (King and McFarland, 2012), including a pilot study at poultry facilities. Particles contained in a large volume of air are concentrated in a relatively small volume of liquid and tested by qualitative or quantitative methods to determine the presence and/or concentration of bacterial pathogens. A wet collection system protects cells from osmotic stress. The aims of this study were to determine the presence of Shiga toxin-producing *Escherichia coli* (STEC) and

Salmonella in the air of meat harvesting establishments using a WWC and to compare the effectiveness of CDx, an immunoassay-based automated system vs. quantitative PCR (RT-PCR) at detecting *Salmonella* and STEC in air samples.

CHAPTER II

LITERATURE REVIEW

Despite the guidelines from the FDA and other related agencies, food safety is still considered a major worldwide health and economic problem. According to the Centers for Disease Control and Prevention (CDC) 2011 report, foodborne pathogens caused 48 million people to become sick, resulting in 128,000 hospitalizations and 3,000 deaths in the United States annually (CDC, 2011). Meat is one of the food products that is implicated in foodborne illness outbreaks. Of the 864 outbreaks of foodborne illness reported in 2014, 80 were linked to the consumption of meat, poultry, dairy, and eggs. Of these, 15 outbreaks were linked to beef, particularly ground beef. *Salmonella* and STEC were two frequent pathogens causing outbreaks linked to beef (CDC, 2016). In spite of all of the improvements made by the beef industry in the reduction of foodborne pathogens, beef continues to be a common food that serves as a vehicle for pathogens that cause foodborne illness. According to the CDC, at least 75 outbreaks were associated with beef over the past five years. Out of these, 35% were caused by *E. coli* O157:H7 and another 23% were caused by *Salmonella* (CDC, 2013b). Since *Salmonella* and *E. coli* were the most common pathogens causing beef-related outbreaks, the present study and this review of the literature are focused on *Salmonella* and STEC.

Sources and mechanisms of transmission of *Salmonella* and STEC to the beef carcass or beef products.

Salmonella and STEC are able to contaminate meat and meat products from different sources. However, animal hides have been found to be a major source of foodborne pathogens,

including *Salmonella* and STEC in beef plants (Barkocy-Gallagher et al., 2003; Bosilevac et al., 2009; Kunze et al., 2008). During the slaughter processing, *Salmonella* and *E. coli* can spread from the hide and transfer into the carcass surface and the plant environment through different mechanisms; consequently contaminating the meat and meat products. The mechanisms of transmission are processing equipment and utensils, and the hand and clothes of the workers during the slaughter processing (Smeltzer et al., 1980). It was suggested that contamination occurs through contact with contaminated surfaces or fecal contamination. But later on, the air has been recognized as a potential source of contamination of beef plants (Burfoot et al., 2006; Sofos et al., 1999b). Another source of contamination of *Salmonella* and STEC in meat plants is the gastrointestinal tract of cattle. Cattle lymph nodes are another source of beef contamination because they are located in fat tissues of the animal and are not removed during fabrication and are ground with beef when making ground beef (Arthur et al., 2008; Small et al., 2006).

2.1 Salmonella

2.1.1. General description of *Salmonella*

Salmonella are facultative anaerobic Gram-negative, non-spore-forming rod-shaped bacteria. They are oxidase negative, catalase positive, non-lactose fermenting, glucose fermentative and belong to family Enterobacteriaceae*.* Salmonellae are widely distributed in nature, humans and animals being the primary reservoirs, and can be found in the environment and the gastrointestinal tract of diverse animals (Pui et al., 2011). They are motile with peritrichous flagella; however, some strains are non-motile (Jay et al., 2005; Andino and Hanning, 2015).

Salmonella are mesophilic and have an optimum growth temperature of 37 ºC; however, they are non-fastidious as they readily adapt to extreme conditions, and grow at temperatures as low as 5.9 ºC and as high as 54 ºC (Odumeru and León-Velarde, 2012; Andino and Hanning, 2015). *Salmonella* are able to survive when exposed to heat treatment, especially in low water activity (a_w) foods. They have been reported for specific experimental conditions, the presence of sigma factors, which are proteins that compose fundamental subunits of prokaryotic RNA polymerase, provides a mechanism for cellular responses by activating genes in response to stress (Kazmierczak et al., 2005). The survival of *Salmonella* in meat and poultry products, especially unprocessed product during frozen storage has been reported (Andino and Hanning, 2015). *Salmonella* uses shock proteins (CSP) to survive low temperatures ranging from 5 °C to 10 °C (Jeffreys et al., 1998; Craig et al., 1998). *Salmonella* can persist in nature and survive long periods of time in dry products (Andino and Hanning, 2015). Many outbreaks have been implicated with dry foods (Li et al., 2012; Podolak et al., 2010). This organism has an optimum growth pH of 6.5-7.5; however, it can grow at pH values of 4.5-9.5. They require high a_w ranging between 0.99- 0.94, but they can survive at a_w less than 0.2 such as low a_w foods (Bhunia, 2007).

Salmonella are divided into two species: *S. enterica* and *S. bongori*. *S. enterica* is also divided into 6 subspecies, and it is the main cause of foodborne salmonellosis. The subspecies include over 2,500 serovars of *S. enterica* that have been classified based on their somatic (O) antigens and flagellar (H) antigens (Jay et al., 2005; Forshell and Wierup, 2006; Malorny et al., 2011). For epidemiological purposes, the salmonellae are placed into three groups including those that infect humans only, which consist of a group of *Salmonella* that causes typhoid and paratyphoid fevers in humans. The typhoid group comprised of *S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi C. The second group is the host-adapted serovars. The strains within this group are

either human or animal pathogens. An example of an animal pathogen is *S*. Dublin, which is a *Salmonella entrica* serotype and is a cattle-adapted bacterium that causes a bloodstream infection in human. The third group in this taxonomy is non-adapted serovars or no host preference. The serovars within this group are causative agents of foodborne illness, and they are both human and animal pathogens (Pui et al., 2011).

Salmonella is one of the leading causes of foodborne illness in the US and causes food poisoning (salmonellosis) resulting from the ingestion of foods containing bacteria. According to the Centers for Disease Control and Prevention (CDC), approximately, 1.4 million people become infected due to human *Salmonella*, resulting in around 16,000 cases and nearly 600 deaths each year in the US (Mead et al., 1999), and these numbers are just accounted for about 2% cases that were reported to CDC (Cummings et al., 2010). The actual number of cases, however, may be more than 30 times larger than reported, considering the underreporting of milder illnesses. Usually, high doses of *Salmonella* are required to cause infection (about 10^6), but depending on the type of foods and condition, the infecting dose may be lower or higher (Kothary and Babu, 2001).

The mode of transmission is the fecal-oral route. The incubation period is 6- 48 hours and salmonellosis symptoms include diarrhea, nausea, fever, abdominal pain, vomiting, and headache. Poultry meats and eggs are the most common food associated with *Salmonella* infection; however, red meats, fruits and vegetables, cheese, chocolates, and milk also have been reported to be associated with *Salmonella* infection (Zhao et al., 2008; Paglietti et al., 2009; Gómez-Aldapa et al., 2012). The mechanism of *Salmonella* infection is still not fully known. Salmonellosis is usually self-limiting; however, in extreme cases, it can lead to systemic infections and chronic conditions (Gomez et al., 1997).

2.1.2. *Salmonella* virulence factors

According to the literature, there are at least 60 genes associated with pathogenicity of *Salmonella*. The *invA, spv*, *fimA* and *stn* genes are major virulence genes responsible for salmonellosis. *Salmonella* has two virulence mechanisms, chromosomal and plasmid factors in combination. These factors are responsible for the virulence of *Salmonella* (Chaudhary et al., 2015; Oludairo et al., 2013; Oliveira et al., 2003). The chromosomal mechanism involves the *invA* gene, which is an important virulence factor for *Salmonella*. This gene is responsible for the invasion of the bacteria into host cells, and it is essential for full virulence in *Salmonella* and establishment of infection in host cells (Sunar et al., 2012; Suez et al., 2013; Oludairo et al., 2013). The invasion gene *invA* codes for a protein that is located in the inner membrane of bacteria, and this gene is required for invasion of epithelial cells (Darwin and Miller, 1999; Suez et al., 2013). The gene *invA* is considered conserved among *Salmonella* serotypes (Oliveira et al., 2003). Therefore, it is found to be appropriate for polymerase chain reaction (PCR) target for *Salmonella* detection (Oludairo et al., 2013; Jamshidi et al., 2008; Chaudhary et al., 2015). The other *Salmonella* virulence factor is the plasmid factor that consists of an operon (spvRABCD), which includes the production of enterotoxin, which aids *Salmonella* ability in promoting the intracellular infection and persistence at extra intestinal sites (Libby et al., 2000, Oliveira et al., 2003). According to some studies, the virulence plasmid plays a significant role in human disease and it contains five genes. The five plasmid genes include *Salmonella* enterotoxin *stn, Salmonella* Enteritidis fimbriae (sef) plasmid-encoded fimbriae (*pef A*) genes, *invE* , *himA* , and *phoP* genes, which is present on plasmids commonly associated with some serotypes (Chiu and Ou, 1996; Rahmanand Dutta, 2003; Chaudhary et al., 2015). Regarding the spv operon, its main function is to potentiate the systemic spread of the pathogen.

The *spvC* is a virulence-related gene on the plasmid required for survival within the host cell (Chiu and Ou, 1996), while the *stn* gene is responsible for mediation in the production of enterotoxin (Chaudhary et al., 2015). Thus, it plays a significant role in causing gastroenteritis by producing enterotoxin (Chopra et al., 1987). The *pef* region or gene is found to be responsible for biosynthesis of fimbriae (Chaudhary et al., 2015).

2.1.3. Presence of *Salmonella* in meat and meat products

The average annual consumption of beef is 55.6 pounds per person in the US (USDA, 2017). However, the contamination of meat products by microorganisms, particularly pathogens, is a major problem in the meat industry. The chemical composition of meat and its fundamental characteristics (a_w above 0.99 and pH between 5.5 and 5.8) make fresh meat a good substrate for growth of microorganisms. Meat can be contaminated with pathogenic microorganisms at various points during the slaughter process, cold storage, and processing of meat animals (Sofos et al., 1999a). Storage at low temperatures (internal temperature $\leq$ 7 °C, within 24-30 hours following slaughter) immediately after slaughter is essential to ensure the microbiological quality of the product (Giaccone et al., 2011). *Salmonella* can contaminate meat due to the presence of this pathogen in the intestinal tract of animals. According to Andino and Hanning (2015), beef products are among the top five foods that have been implicated with *Salmonella* foodborne outbreaks. Greater microbial contamination occurs during the slaughtering process, especially evisceration and dehiding. Hides can be heavily contaminated with pathogenic microorganism through feces (Giaccone et al., 2011; Gómez-Aldapa et al., 2012; Sofos et al., 1999a).

Following good manufacturing practices by the meat industry aids in decreasing the potential contamination of meat products in the meat facility. In addition, decontamination of

carcasses through the application of antimicrobial interventions during slaughter/dressing can also help in controlling pathogenic organisms. An example of a common antimicrobial intervention is the application of organic acids, such as lactic acid or acetic acid. This intervention may assist in reducing pathogen levels, but cannot replace adequate food safety practices during slaughter (Giaccone et al., 2011). In addition, meat and meat products can also be contaminated during various meat processing steps, including the production chain, and after cooking by cross-contamination, which is a common mechanism in the cause of *Salmonella* infection. Therefore, preventing cross-contamination is an important step to help reduce foodborne illness throughout the production chain and after cooking the meat (Giaccone et al., 2011; Andino and Hanning, 2015).

2.1.4. Outbreaks associated with *Salmonella* in meat

Nontyphoidal *Salmonella* is the most common causative agent of foodborne cases in the US. It is estimated to cause approximately 130 outbreaks of foodborne illness involving one million cases every year (Scallan et al., 2011; CDC, 2011). A total of 1,965 of food-related outbreaks of *Salmonella* infection were reported in the US between 1973-2011. Ninety-six of these outbreaks with 3,684 reported cases, were attributed to beef in the US (CDC, 2011). Different types of meat products were involved as sources of outbreaks. Ground beef has been among meat products that served as a vehicle of a large number of outbreaks in the 2000-present. Ground beef was implicated in 17 of *Salmonella* outbreaks, which accounts for 45% of 38 beef outbreaks in the period 2002-2011 (Laufer et al., 2015).

2.1.5. Isolation of *Salmonella* from food

Current procedures for *Salmonella* isolation from foods are based on standard procedures described in the FDA Bacteriological Analytical Manual (BAM) and the FSIS Microbiology Laboratory Guidebook (MLG) (Feng et al., 2017; USDA-FSIS, 2014). The isolation of *Salmonella* is derived from the knowledge that if it is present in foods, it is usually in low concentrations; the cells are in poor physiological condition due to various food processes, and non-homogeneous distribution and storage (Litchfield and Insalata, 1973). The isolation generally includes the pre-enrichment of food samples in a non-selective broth medium, enrichment in a selective broth medium, isolation of presumptive colonies on differential plating media, biochemical and serological testing of presumptive colonies (D'Aoust, 1981). The pre-enrichment of the sample in a non-selective medium prior to selective enrichment is important because it allows the growth of small numbers of *Salmonella* in the presence of large numbers of other organisms. It also allows the resuscitation and recovering of sublethally injured or stressed *Salmonella* cells and growing to detectable levels (Litchfield and Insalata, 1973). For this purpose, many different pre-enrichment media are used. In the FSIS MLG, the recommended pre-enrichment procedure for fresh beef products utilizes modified Tryptone Soy Broth (mTSB) and Buffered Peptone Water (BPW), homogenized with the sample (Jay et al., 2005), followed by incubation for 15-18 h at 35-37 ºC.

Hoorfar and Baggesen, (1998) compared the efficacy of BPW and Universal (UB) pre-enrichment in recovering injured microorganisms. They found that due to the higher buffering capacity of UB compared to BPW, it resulted in greater sensitivity and higher recovery of injured *Salmonella.* In addition, UB contains sodium pyruvate, which is also an important agent for repairing injured microorganisms (Ray and Speck, 1973; Bailey and Cox, 1992). After the

incubation period, the sample is then enriched in an enrichment medium with a selective agent that allows the growth of *Salmonella* and suppresses the growth of other microbiota. The enrichment procedure aids in increasing the probability of isolating *Salmonella* from the sample. For enrichment media, Rappaport-Vassiliadis (RV) broth with 1 ml of pre-enrichment broth and Tetrathionate Brilliant green (TTB) broth with 0.5 ml of pre-enrichment are used. The enrichment medium then is incubated at 35 ºC for 18-24 hours. After enrichment, a sample from the enrichment broth is streaked or spread on a differential or a selective agar medium for *Salmonella* isolation and incubated at 42 ºC for 18-24 h. The agar medium that is used for isolation is usually characterized by containing one or more selective agents. The principle of selective agents is to inhibit Gram-positive, and suppress *Proteus* sp. (Litchfield and Insalata, 1973). Examples of selective media for *Salmonella* isolation are Brilliant Green (BG), Bismuth Sulfite (BS), Xylose Lysine Deoxycholate (XLD), or Xylose Lysine Tergitol 4 (XLT4) Agar. After isolation of presumptive *Salmonella* colonies, biochemical reaction tests are performed for further confirmation of suspect colonies isolated from selective media. Many differential agar tube media are used for confirmation purposes; however, the most common medium that has been specified by FDA and AOAC is the Triple Sugar Iron (TSI) agar, which detects production of hydrogen sulfide and utilization of glucose, lactose, and sucrose. In addition, Urea broth is used to detect the production of the enzyme urease, while Lysine Iron Agar (LIA) is used to determine production of hydrogen sulfide and decarboxylation of lysine. *Salmonella* are lactose negative, urease negative and lysine decarboxylase positive. The biochemical tests are not sufficient for confirmation of presumptive isolated colonies. Therefore, antigenic analysis with serological tests is performed for further confirmation and identification of *Salmonella*. The characterization of *Salmonella* using serological tests is based on the somatic (O) and flagellar

(H) antigens, which involves the use of polyvalent O and H antisera and latex agglutination, using commercially available kits. The procedure is achieved by picking suspected colonies from selective and differential media and mixing with the latex reagent before reading results. A positive result is determined by observing agglutination (Odumeru and León-Velarde, 2012).

The isolation process along with biochemical confirmation and serological testing usually takes between 4-5 days (Downes and Ito, 2001). Therefore, there exists a demand for rapid and reliable methods for *Salmonella* detection in foods and the processing environment in order for the food industry to reduce time and costs. Various rapid immunological and molecular-based methods for detecting of *Salmonella* have been developed (Odumeru and León-Velarde, 2012). Currently, several immunological-based methods are available for detection of *Salmonella* based on antibody-antigen reactions. Enzyme-Linked Immunosorbent Assay (ELISA) is one of the immunoassay methods that is commonly used to detect the presence of an antibody or an antigen in a sample ((Odumeru and León-Velarde, 2012).

Polymerase Chain Reaction (PCR) is a molecular-based approach for rapid detection of foodborne pathogens. This technique is based on amplification of specific or target DNA sequence after DNA is extracted from the sample (Jeníková and Demnerová, 2000). Additionally, a real-time PCR assay has emerged as an important tool in molecular detection of foodborne pathogens in food and environmental samples (Odumeru and León-Velarde, 2012; Zadernowska and Chajęcka, 2012; Özkalp, 2012; Jay et al., 2005). This technique is used to qualitatively and quantitatively detect foodborne pathogens by monitoring DNA amplification using fluorescent signaling (Bustin et al., 2005). In addition to previous examples, there are many other rapid methods available for detection of *Salmonella* in food and the environment

such as nucleic acid hybridization, phage-based detection methods, and multiplex PCR (Odumeru and León-Velarde, 2012).

In *Salmonella* isolation procedures, selective media are used because they only allow growth of target bacteria such as (*Salmonella*) and prevent the growth of non-desired organisms. As mentioned before, the selective media that are used for *Salmonella* include Brilliant Green (BG) agar, Bismuth Sulfite (BS) agar, Xylose Lysine Deoxycholate (XLD) agar and Xylose Lysine Tergitol 4 (XLT4) Agar. Almost all these selective media have a sulfur source, such as sodium thiosulfate, in their formulation, which is used by *Salmonella* to produce H_2S during reaction with iron to form black-centered colonies. *Salmonella is* also able to form the lysine decarboxylase enzyme; therefore, some selective media designed to be used in *Salmonella* detection contain lysine. In XLT4 agar, Tergitol 4 functions as a selective agent, inhibiting the growth of competing bacteria.

2.2. STEC

2.2.1. General description of Shiga Toxin-Producing Escherichia coli (STEC)

Shiga toxin-producing *Escherichia coli* (STEC), also called verotoxin-producing *Escherichia coli* (VTEC), is a group of pathogenic *E. coli* that has the ability to produce one or two verocytotoxins (Shiga toxins) that cause illnesses in humans called Shiga toxin (*Stx1*) and (*Stx2*) (Blabco et al., 2003; Lim et al., 2010; O'Sullivan et al., 2007; Auvray et al., 2007). STECs were first recognized as foodborne pathogens in 1982 (Riley et al., 1983; Auvray et al., 2007). STECs are Gram-negative, facultative anaerobic, rod-shaped bacteria. They are non-spore-forming and some strains are motile with peritrichous flagella. They are natural inhabitants of the gastrointestinal tracts of some ruminants. Environmental temperature is a significant factor of

growth and survival of STEC. The optimum temperature for growth of STEC is approximately 37 °C; however, STECs have the ability to replicate in temperatures as low as 7 °C and as high as 50 °C (Jay et al., 2005). When exposed to high temperatures, STEC experience some physiological changes. For example, the structures of cell membrane phospholipids are altered with increasing growth temperatures (de Mendoza and Cronan, 1983). Exposure to sublethal heat can also activate mechanisms for thermal resistance, which make organisms more resistant to future thermal processing (Juneja et al., 1997).

Cattle are the main reservoir of STEC, introducing a major risk of contamination in meat, dairy and produce. Beef has been implicated in many STEC infection outbreaks, primarily associated with undercooked ground beef (Blanco et al., 2003; O'Sullivan et al., 2007). Furthermore, many other foods have been linked to STEC infection such as unpasteurized milk and fruit juice, lettuce, spinach, cantaloupe, cheese, mushrooms, and sprouts (O'Sullivan et al., 2007). The most prevalent STEC serotype is *E. coli* O157:H7; the six non-O157 STEC serogroups are O26, O45, O103, O111, O121, *and* O145 (Brooks et al., 2005).

While *E. coli* O157:H7 is the causative agent in most reported cases of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), humans may be more likely to be exposed to non-O157 STEC due to the prevalence of those serotypes in cattle. (Blanco et al., 2003; Karmali. 1989). The infection with non-O157 STEC types, such as O26, O45, O145, and O121, O111, and O104 has also been associated with severe illness in humans (Blanco et al., 2003) and it has been suggested that 20 to 50 % of *E. coli* outbreaks are due to non-O157 strains (Hughes et al., 2006). In 2006 an outbreak of *E. coli* O26 was reported at Niigata City Health Center that implicated food served at a restaurant (Miyajima et al., 2007). Another outbreak of HUS of non-O157, specifically *E. coli* O111 was also reported in 1992 and was implicated with ground beef

(Caprioli et al., 1994). An outbreak of STEC with over 4000 cases and over 50 deaths occurred in Germany in 2011, and was caused by *E. coli* O104:H4. The food implicated in this outbreak was fenugreek sprouts (Scheutz et al., 2011; Beutin and Martin, 2012).

Infection with STEC can range from asymptomatic cases to death. In most cases, the infection causes non-bloody diarrhea and self-resolves without complication. However, in severe cases, the infection can result in HUS. HUS is a serious condition that can sometimes be fatal, as it can involve bleeding, anemia, and kidney failure (Lim et al., 2010). The symptoms of STEC infection include diarrhea that may be bloody, abdominal pain, nausea and vomiting (Yoon et al., 2013, usually lasting for 5 to 10 days. Symptoms are more severe in children, the elderly and people with a compromised immune system (Karmali, 2004; Kim et al., 2005).

Avoiding eating undercooked meat, unpasteurized milk products, and washing hands thoroughly after handling animals, changing diapers, and before eating can significantly prevent or reduce STEC infection. Proper food storage, handling, and keeping raw foods separate from cooked foods are also important to prevent cross-contamination (CDC, 2013c). STEC can survive and persist in various environmental conditions such as soil, water, food products, and cattle. *E. coli* O157:H7 can survive in a soil environment for one year, and for a long time in water, especially at cold temperatures (Lim et al., 2010). Wang and Doyle (1998) were reported that *E. coli* O157:H7 can survive for 91 days in cold water. The minimum growth temperature for STEC is 7-8 °C and the maximum is 44-46 °C with optimum growth at 35-40 °C. STEC is heat sensitive and can be destroyed completely by typical thermal processing of food. The optimum pH for growth of STEC is 6.5-7.0. Depending on the strains, *E. coli* O157:H7 can stand acidity as low as pH 2.5 for acids. *E. coli* is more resistant to acid than most of the Gram-

negative pathogenic bacteria. Finally, the optimum a_w for STEC growth is 0.995. (Lim et al., 2010).

2.2.2. STEC virulence factors

The major virulence factor and mechanism of STEC pathogenesis is the production of Shiga toxins (*stx*). Shiga toxins are strong cytotoxins that are bacteriophage-encoded. These toxins can expand from a single transcriptional unit and cause damage to a variety of cells (Jacewicz et al., 1999; Lim et al., 2010).

Shiga toxins consist of two main categories (*stx1* and *stx2*) and are encoded by *stx1* and *stx2* genes. Both *stx* types have an AB5 structure in which a single A-subunit is associated with five B-subunits.

stx1 is identical to the Shiga toxin of *Shigella dysenteriae,* and *stx2* is less related to the Shiga toxin of *Shigella* (Boerlin et al., 1999). The virulence factor could be *stx1* only, *stx2* only, or both toxins. The *stx2* is known to be more toxic and is more often associated with HC or HUS in human infections than are *stx1* (Lim et al., 2010). Both *stx1* and *stx2* are responsible for blocking protein synthesis in eukaryotic cells ((Boerlin et al., 1999). The *eae* gene encoding the intimin protein is also considered a virulence factor, responsible for the adhesion and colonization of STEC strains onto the intestinal epithelium of the host (Loukiadis et al., 2006). The *eae* gene that is an encoded outer membrane protein, contributes to STEC pathogenesis through mediating adherence of the bacteria to host intestinal epithelial cells and forming attaching and affecting lesions. In addition, a plasmid-encoded enterohemolysin (*ehxA*) are also virulence vectors in STEC (Lim et al., 2010; O'Sullivan et al., 2007). And the role of enterohemolysin (*ehxA*) gene is to enable STEC strains to cause disease. The plasmid-encoded

enterohemolysin is present in both STEC O157 and non-O157 strains (Lim et al., 2010; O'Sullivan et al., 2007).

Other virulence factors are a group of genes within the pO157-encoded plasmid; include a catalase-peroxidase (katP), an extracellular serine protease (espP), a zinc metalloprotease (stcE), a subtilase cytotoxin (subAB), and putative adhesion (toxB) (Lim et al., 2010). The pO157-encoded plasmid is highly conserved, and it is a non-conjugative F- like plasmid with a size ranging from 92 to 104 kb. This plasmid is an extrachromosomal DNA that is capable of replicating independently of the chromosomal DNA. It is responsible for providing resistance to antibiotics and heavy metals, production of toxins and other virulence factors, biotransformations of hydrocarbons, and symbiotic nitrogen fixation ((Lim et al., 2010).

2.2.3. Presence of STEC in meat and meat products

Animals have been identified as a major reservoir of STEC (Karmali et al., 2010). Thus, carcasses and resulting meat products can become contaminated with these microorganisms during processing through contact with intestinal contents or from the hide, as well as cross-contamination by equipment or workers (Edwards and Fung, 2006). Masana et al., (2010) studied the prevalence of STEC in feces and carcasses of cattle from beef exporting abattoirs of Argentina and showed that non-O157 STEC was present in 22.3% and in 9.0% of feces and carcasses, respectively, whereas the prevalence of O157 STEC in feces and carcasses was 4.1% and 2.6% respectively. The prevalence of non-O157 STEC in beef cattle feces was reported to range from 9 to 30% and was found on 56.3% of samples hides (Barkocy-Gallagher et al., 2003). Ground beef has been linked to many STEC outbreaks and is considered to be one of the main sources of STEC infection in humans (Robbins et al., 2014). Bosilevac and Koohmaraie (2011)

studied the prevalence of STEC in ground beef in the US and reported that 7.3 % of ground beef samples tested were positive for STEC non-O157.

2.2.4. Outbreaks associated with STEC in meat

According to a CDC report, 1 in 6 Americans, or approximately 48 million people acquire a foodborne illness in the US annually, resulting in 128,000 hospitalizations and 3,000 deaths (CDC, 2011). Most of these cases were caused by foods of animal origin, with about 18.0% cases linked to dairy, 17.9% to poultry, and 13.2 % attributed to beef and beef products (Painter et al., 2013). Each year, about 7.4% of hospitalization cases and 5.9% of deaths are caused by contaminated beef (Painter et al., 2013).

STEC is one of the causative agents of foodborne illness. STEC can be transmitted through different routes, including consumption of food and water that has been contaminated via feces, from person to person, or from animal to person. In recent years, the potential for airborne transmission was also reported as a source of contamination (Dunn et al., 2004; Lim et al., 2010; Varma et al., 2003).

Many outbreaks are linked to STEC infection in the US. A total of 183 outbreaks was caused by STEC, specifically *E. coli* O157:H7 during the period between 1982-2002 in the US. In most of these outbreaks, the common source of infection was ground beef. Out of 183 outbreaks mentioned above, seventy-five outbreaks were linked to ground beef (Rangel et al., 2005). A large multistate outbreak of *E. coli* O157:H7 infections occurred in the US between 1992 and 1993. This outbreak was associated with the consumption of undercooked hamburger and caused over 700 cases of foodborne illness and 4 deaths (Bell et al., 1994, 1997; Jay et al., 2004). Two other outbreaks that also were linked to *E. coli* O157:H7 caused by consumption of

minced beef were reported in the 2000s (Mailles et al., 2006). Usually, undercooked beef is found to be the reason for most of the outbreaks associated with *E. coli* (Orr et al., 1994; Macdonald et al., 2000; Macdonald et al., 2004; Laine et al., 2005). Currie et al. (2007) investigated beef donair, which is made of a mix of ground beef and spices that is put into a cone, and then cooked in a vertical broiler. These authors found beef donair product to be a potential risk factor for *E. coli* O157:H7 infection because they identified inadequate cooking and handling of the product during cone formation and long cooking periods. While most of the outbreaks of *E. coli* O157:H7 infection have been linked to meat or meat products, many studies have shown that the prevalence of non-O157 STEC is similar to the prevalence of *E. coli* O157:H7 in beef and beef products (Samadpour et al., 1994; Brooks et al., 2001; Samadpour et al., 2002; Bethelheim et al., 2003; Fratamico et al., 2011; Monaghn et al., 2001). Since meat and meat products was identified as a vehicle of foodborne illness especially with *E. coli* O157:H7, the US Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) required all meat and poultry establishments to develop, institute, and keep record of sanitation standard operation procedures, and also implement generic *E. coli* testing to verify process controls by slaughter establishments, and develop and implement Hazard Analysis and Critical Control Point (HACCP) systems (FSIS, USDA, 1996). Furthermore, The USDA-FSIS declared and considered raw ground beef to be adulterated if contaminated with *E. coli* O157:H7, and if control measure is not applied to eliminate pathogens (FSIS, USDA, 1996).

2.2.5. Isolation and Detection of STEC

Due to levels of injured cells with high concentration of microbiota, non-homogenous distribution of target pathogens, and complex food composition and environmental matrices, the

isolation of STEC with direct cultural plating is difficult (Ge et al., 2002). Therefore, the detection of *E. coli* O157:H7 strains from food samples requires enrichment and isolation with selective and/or indicator media. Enrichment is used for STEC isolation to recover injured or stressed cells, increase the target cell numbers, and dilute the effect of food and environmental inhibitors and microbiota in the sample (Wang et al., 2013). However, the specificity of the STEC strains can be used for their identification, as it is difficult to isolate these strains using conventional methods. This is due to the fact that these microorganisms do not have differential phenotypic characteristics that can differentiate them from non-pathogenic *E. coli* strains. In the laboratory, the STEC strains are distinguished from non-pathogenic *E. coli* based on a unique phenotypic trait (Gilmour et al., 2009). In addition, there is no single method to isolate or detect all STEC strains. The conventional culturing methods rely on biochemical tests. Therefore, more sensitive methods are required to isolate and identify STEC from the food samples (Kim et al., 2005). For conventional culture methods, several commercial agars are available. Almost all available media are based on sorbitol fermentation because most O157 strains do not ferment sorbitol; however, the differentiation of non-O157 STEC from other *E. coli* strains is still not easy. Recently, Possé agar has been used for STEC isolation. This medium is based on a mixture of carbohydrates (sucrose and sorbose) for ß-D-galactosidase activity, and selective compounds (bile salts, novobiocin, and potassium tellurite). Possé agar was used for color-based differentiation of STEC (Possé et al., 2008). The isolation procedure for STEC is based on the protocol described in the FDA BAM and the FSIS MLG (Feng et al., 2017; USDA-FSIS, 2014). The isolation consists of multiple incubation steps that include pre-enrichment, selective enrichment, selective and differential plating for isolation of STEC followed by biochemical and serological tests (Ge and Meng, 2009). Recently, USDA has done some improvements in the

selective enrichment media formulation that used of *E. coli* O157:H7 isolation; such as using some antibiotic. Currently, pre-enrichment for *E. coli* O157:H7 identification, mTSAB broth (modified tryptone soy broth with novobiocin and casamino acids) is used instead of mEC broth (Ge et al., 2002). The pre-enrichment consists of placing of 25 g of food or environmental sample in 225 ml of enrichment broth, such as mTSB for 15-18 h, then streaked and surface plated onto rainbow agar or onto Possé agar, which is MacConkey based agar overlaid with non-selective agar (TSA) to recover sublethally injured cells and incubated at 37 °C for 24 hours. Then biochemical tests are performed for further confirmation. Further confirmation is performed using serological tests such as O157 antigen latex, followed by H7 antiserum test. Biochemical tests are used for identification of *E. coli* species.

For rapid detection of STEC, several techniques are available. The immunomagnetic separation method is used, which is utilized magnetic beads coated with antibodies specific for the O-antigens of STEC strains (Auvray et al., 2007). PCR molecular-based methods are also used for detection of *Stx* (*stx1* and *stx2*), which is based on the detection of *stx* genes in the food and environmental samples (Perelle et al., 2004; Gilmour et al., 2009; Kistler et al., 2011). Recently, a real-time PCR (RT-PCR) approach was developed to detect the presence of *stx1* and *stx2* genes quantitatively and qualitatively. RT-PCR has exhibited high sensitivity and specificity in detecting *stx1*, *stx2*, and *eae* genes (Anklam et al., 2012; Verstraete et al., 2014).

STEC can be characterized by serological testing (slide agglutination), which is based on the H7 antiserum and O175 antiserum for STEC. Chromogenic agar can be used to differentiate different STEC strains based on color reaction. The color reaction is based on whether organisms ferment sorbitol within 24 hours (non-O157) or not ferment sorbitol (O157). Possé agar can also differentiate between different STEC isolates based on color. Using DNA technology with

conventional gel-based PCR application (Auvray et al., 2007; Kim et al., 2005), and colony hybridization assay is another technique that can be used to characterize STEC strains as described previously (Fach et al., 2001).

Currently, the most widely used selective medium for the detection of non-sorbitol fermenting *E. coli* O157:H7 is sorbitol MacConkey (SMAC) agar. The selectivity of SMAC media is improved by the addition of selective supplements cefixime and potassium tellurite (CT-SMAC) (Zadik et al., 1993). However, this medium is not suitable for the detection of non-O157:H7 and sorbitol-fermenting *E. coli* O157:H7. (Mathusa et al., 2010). Interestingly, there are no standard methods for isolation of non-O157 STEC from foods. The lack of recognized physiological characteristics between non-O157 STEC and other non-pathogenic *E. coli* strains is a reason that makes isolation and detection of STEC difficult (Mathusa et al., 2010; Mingle et al., 2012). Therefore, different media have been developed and tested for detection and isolation of STEC. Recently, several chromogenic media have been developed commercially for detection of *E. coli* O157:H7. The principle of these media is based on the production of the different colors in the growing colonies resulting from reactions other than sorbitol fermentation. Instead of being based on sorbitol fermentation, these media contain a certain mixture that contains artificial chromogenic. These chromogenic substances are degraded by specific enzymes and release differently colored compounds by different strains of STEC to generate different colony colors. The chromogenic agars are also based on characteristic traits such as sorbitol fermentation, glucuronidase or galactosidase activity and are effective for *E. coli* O157:H7 isolation. Examples of these agars include; CHROMagar ^{TM} O157:H7 (CHROMagar Microbiology, Paris, France), and PARID' E. coli^{TM} O157:H7 (Biorad, Hercules, CA, USA). A rainbow agar (Biolog, Hayward, CA, USA) is another medium that has been widely used for

STEC isolation. The selectiveness of this medium is based on the reduction of tellurite to tellurium. Therefore, potassium tellurite is added, which is highly selective for *E. coli* O157:H7; this component inhibits the growth of other microbiota (Tilman et al., 2012). However, false positives may occur because of the presence of other microorganisms that able to reduce tellurite. The microorganisms such us *Proteus* can also reduce tellurite to tellurium. Therefore, novobiocin will be added to inhibit tellurite-reducing bacteria (Hussein and Bollinger, 2008).

The chromogenic media that are used for *E. coli* O157:H7 are not very useful for the non-O157 isolation due to the variability of phenotypic characteristics of non-O157. A set of novel differential media for the isolation and confirmation of *E. coli* O157:H7 and non-O157 strains particularly (O26, O103, O111, and O145) have been developed by Possé et al. (2008). The four different non-O157 STEC serotypes on this medium produce different colored colonies on this medium. *E. coli* O26 produces bright red to dark purple colonies, O103 and O111 colonies are blue-purple and O145 colonies are green. . These agars contain phenol red broth base supplemented with dulcitol, L- rhamnose, D-raffinose or D-arabinose (Mathusa et al., 2010). Rainbow O157 agar has also been utilized as selective media for the detection of non-O157 serotypes. However, a modified version of this media was used usefully to isolate STEC. This modified version media contained 0.05 mg/L cefixime, 0.15 mg/L potassium tellurite, and 5 mg/L novobiocin that support the growth of various STEC strains.

2.3. Comparison between microbiological detection methods (conventional, rapid methods)

The analysis of food and the environment for the presence of foodborne pathogens and spoilage organisms is a standard technique to ensure safety and quality (Mandal et al., 2011).

Two detection methods are available for detecting microorganisms: conventional testing methods for quantitative detection and rapid automated methods for qualitative or a combination of qualitative and quantitative detection purposes. Noll et al. (2015) compared culture-based and molecular-based methods and suggested for accurate results both methods are necessary for analysis of foodborne pathogens.

2.3.1. Conventional culture methods

Conventional microbial culture methods of sampling food and environmental samples for the presence of microorganisms depend on specific media to enumerate and isolate viable cells in foods and the environment. These methods can provide the number and nature of microorganisms in food and the environment. Usually, the confirmation includes biochemical and/or serological identification tests (Kim et al., 2005; Järvinen et al., 2009).

Conventional methods of detection and isolation of microorganisms are reliable and efficient, they are easy to conduct, low cost, and usually do not require specific skills (Adzitey et al., 2013). However, they are labor intensive and relatively slow, they require several days to weeks before results are produced compared to modern rapid detecting methods, such as molecular-based methods example RT-PCR or immunoassay-based methods such as an ELISA. In addition, phenotypic characteristics by which the bacteria are identified may not be always specified, and when specified, they may be difficult to interpret and classify (Keramas et al., 2004; Myint, 2006).

Other disadvantages of conventional cultural methods are that those methods cannot detect non-culturable organisms in the samples; in one study the percentage of culturable cells without using enrichment steps in the indoor environment was reported as low as 0.03 (Rintala et

al., 2008). In addition, they are less sensitive compared to rapid methods and more susceptible to contamination and human errors. These methods usually are done by human and group work, which result in error and contamination. Meanwhile, rapid methods are mostly done by automated devices (Farzan, et al., 2007; Fu et al., 2006; Kim et al., 2005; O'Sullivan et al., 2007; Sidari and Caridi, 2011; Sunar et al., 20012; Uyttendaele et al., 2003). The detection limit of the selective culture method is about 10^2 CFU/g (O'Sullivan et al., 2007).

In regard to rapid automated methods, there is continuous development of these techniques and reliable detection of foodborne pathogens. Improvement in the molecular-based assay and immunoassay techniques have resulted in the development of faster, more sensitive, and more convenient methods than conventional methods in testing for foodborne contamination of foods and the environment (Mandal et al., 2011).

2.3.2. Polymerase Chain Reaction (PCR)

In this regard, Polymerase Chain Reaction (PCR) is one of the most reliable rapid assays that qualitatively detect microbes in foods and the environment based on nucleic acid (DNA) amplification (Adzitey et al., 2013). Due to its reliability and high sensitivity, PCR becomes a promising alternative approach in food and environmental analysis. It is widely used for detecting pathogens in foods and the environment, especially when particular pathogens are difficult to culture or require a long time to recover and be seen on the media, and the concentration of microorganisms is low (Yamamoto, 2002, Järvinen et al., 2009). However, the analysis with PCR has many disadvantages due to the susceptibility of PCR to inhibitors, contamination, and experimental conditions (Cohen et al., 1996; Yamamoto, 2002).

2.3.2.1. Real-Time PCR (RT-PCR)

The concentration of microorganisms in the environment is low, and at the same time, the information about the level of these microorganisms is needed. Recently, quantitative real-time fluorescence-based polymerase chain reaction (RT-PCR) tests have been developed in microbiology laboratory for the rapid detection and quantitation of pathogenic bacteria in clinical, food, and environmental specimens, allowing early, sensitive, and specific detection of microbes (Bustin et al., 2005; Maurin, 2012; Verstraete et al., 2014). It is a DNA based technique that combines amplification and detection stages of the process to monitor and record DNA amplification continuously (Singh and Mustapha, 2014).

The detection of the RT-PCR products is accomplished via the generation of a fluorescent signal. This fluorescent signal is used to monitor the level of amplification at each cycle. For this purpose, two methods are used. The first method includes fluorogenic probes (TaqMan) that light up to show the amount of DNA present at each cycle of PCR. This process refers to as "Quantitative PCR", which refers to the ability to quantify the starting amount of a specific sequence of DNA. Another approach involves the use of the DNA intercalating fluorescent dye SYBR Green, which is based on binding the fluorescent dye to double-stranded deoxyribonucleic acid (dsDNA) (Valasek and Repa, 2005). Compared to conventional PCR, RT-PCR does not require post-PCR processing, allows straightforward comparison between RNAs that differ widely in their abundance, it permits to better determine the amount of starting DNA in the sample before the amplification by PCR, and it provides quantitative as well as qualitative analysis (Bustin et al., 2005; Valasek and Repa, 2005). Compared to conventional culture methods, RT-PCR provides shorter time, rapid, and excellent analytical sensitivity and selectivity (Singh and Mustapha, 2014). It is both a qualitative and quantitative approach, and it

employs a high degree of automation to reduce the number of operations and the risk of contamination. In comparison to immunoassay, such as ELISA, RT-PCR saves both the total time from sampling to result and time needed to set up and run the assay (Day et al., 2009; Fu et al., 2006; Kim et al., 2005; Odumeru and Leon, 2012; O'Sullivan et al., 2007; VanGuilder et al., 2008; Sidari and Caridi, 2011; Uyttendaele et al., 2003; Wong and Medrano, 2005; Zhang et al., 2011). For example, a typical immunoassay-based detection method requires a secondary enrichment stage before low numbers of cells can be detected and takes up to 48 hours to achieve a meaningful result. The high automation of RT-PCR systems results in minimizing staff time and training. In addition, RT-PCR requires fewer repeat tests than ELISA immunoassay and has a low detection limit, which is ranging 2-10 CFU/ml (Day et al., 2009; Uyttendaele et al., 2003; Zhang et al., 2011). However, RT-PCR is more expensive than traditional plating due to the use of expensive equipment and reagents; also it needs qualified and skilled staff, and because of its high sensitivity, and experimental design, a good understanding of normalization techniques is essential for accurate results. The lack of standardized protocols in RT-PCR impairs its practical implementation for environmental monitoring and control. The long amplicon size decreases the efficiency of RT-PCR; the amplicon size should not exceed 150 bp with an average size between 10-150 bp (Wong and Medrano, 2005).

2.3.2.2. BAX PCR System

The BAX system is another detecting technique, which is an automated molecular pathogen detection analysis that uses PCR technique for detection of pathogens in food and the environment. It features two types of testing technologies including real-time and end point detecting. The system identifies and amplifies DNA fragments for target microorganism. The

targeted DNA is combined with DNA polymerase, nucleotides, and primers. The mixture then undergoes a series of steps similar to PCR amplification. After DNA amplification, the BAX system starts a pathogen detection phase by measuring the fluorescent signal (Tice et al., 2009). In addition, it allows testing multiple targets in the same run. It is a fast, accurate method with high sensitivity and specificity for detection pathogens from food and environmental samples. Furthermore, the BAX system is superior to PCR as it combines all the reagents required for PCR in a single lyophilized tablet, thus it reduces the time required for reagent transfers and the potential for technical mistakes and cross-contamination. In addition, it provides an automated PCR assay by producing and detecting the end product with photometric means (Bailey and Cosby, 2003). Frausto et al., (2013) evaluated the efficiency of the BAX system for detecting of *Salmonella* in chicken meat and found this system is a good alternative method for laboratories, especially for laboratories that analyze a large number of samples. However, positive samples should be confirmed by the conventional culture method.

2.3.3. Immunoassay-based methods

These methods are based on antigen-antibody interaction, which use specific binding reaction between antibodies and antigens. However, the positive results from these methods always require further confirmation with conventional culture methods (Jasson et al., 2010). The detection limit is ranging between 10^4-10^5 CFU/ml, depending on the type of antibody and enrichment process (Jasson et al., 2010). Numerous immunoassays are available for detection of pathogens in foods and environmental samples, such as ELISA, Enzyme-Linked Fluorescent Assay (ELFA), and Immunomagnetic Separation (IMS) assays.

ELISA is one of the immunoassay methods that is widely used for detection of pathogens in food and environment analyses. It reduces the time of detection after enrichment, compared to conventional culture methods; the result can be ready in 2-3 day including enrichment time (Leon-Vrlarde et al., 2009). This method combines immunoassay with an enzymatic reaction, which is used to detect viable cells.

The analysis of food and environmental samples for detecting and separating the organism of interest is one of the most challenging problems due to the complex food compositions and high presence of background debris. Concentration and separation is one of the techniques that can be utilized to overcome these problems. Immunomagnetic separation (IMS) has been developed to accomplish this in rapid and conventional methods (Mandal et al., 2011; Yoshitomi et al., 2012; Chifiriuc et al., 2017).

IMS is a laboratory technique that can efficiently isolate cells from food and environmental samples. In this method, antibodies coating paramagnetic beads are bound to antigens present on the surface of cells, capturing the cells and facilitating the concentration of these bead-attached cells. IMS is frequently used to concentrate the target pathogen directly from food or the environment (Mandal et al., 2011; Chifiriuc et al., 2017).

The concentration process of the target is achieved by utilizing a magnetic “bead” combined with target-specific antibodies. The separation process consists of two steps. In the first step, a magnet is used to immobilize the magnetic particles complexes with the target against the vessel wall, and the remainder of the material is removed. In the second step, the magnetic complex is washed to remove food material and other microorganisms while the particle–target complex is retained. The target then can be detected using conventional immunoassays, streaking or plating onto agar culture media, or by molecular-based methods.

(Mandal et al., 2011; Yoshitomi et al., 2012). Recently, when the application of IMS put together with PCR assays, the outcome results were very promising for the detection of different pathogens. Hudson et al. (2001) used IMS and then PCR to detect *L. monocytogenes*, and they found that the detection limit was 1 CFU/1-25 g of sample. IMS is analog to selective cultural enrichment because it allows the growth of pathogen of interest and suppresses the growth of other bacteria (Mandal et al., 2011).

Many different target organisms of interest can be isolated using this technique, including fungal, bacterial cells or spores, protozoan parasites, cellular and subcellular material, proteins, and nucleic acid products (Yakub and Stadterman-Knauer, 2004). One of the advantages of using IMS, it offers an alternative approach to the rapid identification of culturable and non-culturable microorganisms.

2.4. **The challenges of analyzing food and environmental samples**

Various factors interfere with the detection of microorganisms in all of the detecting techniques, which make the analyses challenging. Among the challenges of analyzing food and environmental samples are the biological stresses of microorganisms during the sampling process (differences in temperature, pH, dry condition, air), which may cause the microorganisms to be sublethally injured. Injured bacteria are usually not detected by conventional methods if the cells are not repairing.

In addition, low bacterial concentration and the heterogeneous distribution of low levels of pathogens lead to failure in the detection of pathogens of interest by traditional methods and most of the rapid methods as well (Mandal et al., 2011; Sidari and Caridi, 2011). The presence of viable but non-culturable organisms, which are not detected by cultural methods, can cause false

negative results (Sunar et al., 2012). The presence of residual compounds in the environmental sample can also inhibit enzymatic reactions and interfere with detection procedures with both PCR and RT-PCR, which result in the decrease of specificity and sensitivity of PCR (Day et al., 2009; Schrader et al., 2012). The complexity of foods and environmental matrix are also important factors. Examples of environmental samples include samples from soil, water, and air. Each of these environmental matrices contains many different inhibitors that affect the PCR and qPCR performance. Among the PCR inhibitors that present in the soil are humic and fulminic acids, and polysaccharides, metal ions, fats, lipids, proteins, polyphenols, dead biomass in water, air and sewage (Schrader et al., 2012; Wilson, 1997).

In addition, the environmental condition such as temperature, pH, humidity, and presence of oxygen all affect the microorganisms' detection by different means and methods. Furthermore, one of the most challenging problems in food analysis is sample preparation. The improper sample preparation may result in cross-contamination during sampling, handling, and analysis of the sample.

Finally, levels and types of background microflora that are present in environmental samples play a significant role in detection techniques.

2.5. Possible solutions to improve and enhance pathogen recovery

To overcome some of previous limitations and challenges in general, certain procedures and techniques are needed. One of the techniques that improve both specificity and sensitivity of detection methods is enrichment as the first step of sample analysis.

This procedure allows the propagation of the pathogen of interest while inhibiting the competitive microbiota. Enrichment also dilutes the effects of inhibitors (Schrader et al., 2012).

In addition, enrichment helps to increase the number of a pathogen of interest by allowing the repair of sublethally injured organisms. However, the enrichment is performed when the aim of analysis is the detection of the pathogen, whereas if the aim of the analysis is counting the pathogen, the enrichment cannot be applied.

For problems related to PCR assay, the detection can be improved in several ways. For example, when using RT-PCR for pathogen enumeration, antibiotics are added to the growth medium to improve specificity and sensitivity (O'Sullivan et al., 2007).

Another approach to maximize the sensitivity of PCR assay or other similar methods is by reliable sample preparation. The reliability of the sample preparation includes but is not limited to precise DNA extraction, as this process is important to obtain enough PCR fragments and good results, while also preventing cross-contamination during DNA extraction and sample preparation of PCR.

Regarding the low cell numbers of pathogens in food or the environment, using separating and concentrating microorganisms in the sample increases the number of organisms in the sample to differentiate the target pathogen from other cells.

For the separation and concentration of pathogen, several methods have been developed. Among these methods is using IMS, The methods include using antibody-based, physical, and chemical-based methods for separation and concentration of pathogens from various sample matrices (O'Sullivan et al., 2007; Sidari and Caridi, 2011).

For conventional methods, the specificity of detection increases by using more than one enrichment broth, increasing the concentration of enrichment and decreasing its volume by using double strength enrichment. In addition, using positive and negative control can increase the sensitivity. Overlay agar method is used to improve culturing media. This procedure helps

recover sub-lethally damaged cells and decreases false negative results (O'Sullivan et al., 2007). Also, selectivity of plating agar and addition of antibiotics result in increased sensitivity; for example, it was suggested that using Cefixime-Tellurite Sorbitol MacConkey (CT-SMAC) for STEC plating increased in sensitivity because the selectivity of this media resulted in better inhabitation of microflora in the sample (Sidari and Caridi, 2011). Furthermore, preventing contamination by applying aseptic technique during sampling and plating improves sensitivity.

For immunoassay methods, using the double strength selective broth increases the sensitivity. Since these methods are also susceptible to contamination, proper handling of the sample and preventing contamination during detection process increases the sensitivity of testing. In addition, immunoassay methods are subjected to cross-reaction with other antigens other than the targeted antigens (antigen sharing) that are present in the sample. Therefore, the use of selective enrichment is required to increase the specificity, and it requires very careful selection of specific antibody-coated antigens. For example, somatic (O) antigens are used in order to improve the specificity (Farzan et al., 2007; O'Sullivan et al., 2007).

2.6. Characterization and identification of microorganisms

Previously, characterization and identification of microorganisms depended on the morphologic and the phenotypic characterization of microbial strains or typical isolate (Clarridge, 2004). In 1980 decade, Woese et al. (1985) and Woese (1987) determined that phylogenetic relationships of bacteria could be identified by comparing a conserved part of the genetic code. The 16S rRNA gene is the conserved part of the bacterial DNA, which is present in 5S of the bacterial ribosome. Currently, the 16S rRNA gene is the most common gene for identifying bacterial taxonomy (Bottger, 1989; Harmsen and Karch, 2004; Kolbert and Persing,

1999; Palys et al., 1997). The 16S rRNA gene has both variable and conserved regions, and sufficient polymorphisms to provide a differentiating and valid measurement for bacterial identification (Clarridge, 2004). Thus, 16S rRNA gene is the most important gene, and is considered universal in bacteria, which can be used to identify the relationships among all bacteria (Woese et al., 1985; Woese, 1987). In addition, the comparison of the 16S rRNA gene sequences provides differentiation between organisms at the genus level across all phyla of bacteria, and also allows classification of strains at multiple levels (Clarridge, 2004). In this regard, sometimes the whole DNA sequence is needed for the identification and characterization of microorganisms, which is called the whole genome sequencing, to differentiate between specific taxa or strains. Sometimes shorter sequences in 16S rRNA region are needed to provide comparable information for differentiation (Sacchi et al., 2002a; Sacchi et al., 2002b). Therefore, 16S rRNA gene is an excellent gene for identifying an unknown organism and bacterial taxonomy (Clarridge, 2004). The 16S rRNA for bacterial identification is used through nucleic acid sequencing methods, which have gone through large stages of improvement over the past decade. These rapid advances have helped the researcher to determine the sequences of millions of base pairs of DNA per year. In addition, it makes it possible to generate a sequence almost in the same day or less than 1.5 days using a colony or specimen (Cook, 2003; Tang et al., 1998). Several methods for DNA sequencing are available, and usually, the procedure starts with bacterial genomic DNA extraction from whole cells using either a standard or available commercial methods (Sambrook et al., 1989). The extracted DNA is used as template for PCR to amplify fragment of 16S rRNA gene sequence using universal primers complementary to conserved regions. The PCR products are then purified using available commercial kits. The purified PCR products are used for DNA sequencing in a process called cycle sequencing. This

process is similar to PCR, and uses both forward and reverse sequences as a template in separate reactions. But it differs from PCR in that it uses either forward or reverse primer, and no new a DNA template is formed during this process. The generated DNA sequences are assembled by aligning the forward and reverse sequences. Finally, the result is compared with the world Wide database library (GenBank) using specific analysis software (Clarridge, 2004). Today, microbial whole genome sequencing carries huge promises to improve diagnostic and public health microbiology.

2.6.1. DNA sequencing and next generation sequencing

DNA sequencing has been widely used for bacterial detection and identification. As described previously, DNA sequencing is performed after a purified PCR product is obtained from the extraction of DNA (Barghouthi, 2011). DNA sequencing is the process to determine the precise order of four bases of nucleotides (adenine, guanine, cytosine, and thymine) within a DNA molecule by different methods or technology (Felske, 1998). The sequencing process provides information about the type of genetic information in a particular DNA segment. Next generation sequencing (NGS) is a promising technology with a high-throughput method that used to determine a portion of the nucleotide sequence of an individual's genome. The NGS utilizes technologies that are enabling of processing multiple DNA sequences in parallel (Mayo et al., 2014). The NGS can be used in two ways; sequencing the total DNA, which is called shotgun sequencing, and gene-specific sequencing called targeted sequencing. Either method provides a higher number of nucleic acid sequences and allowing a deeper description of the microbial constituents of the ecosystems compared to previous generation (Mayo et al., 2014).

Current commercially available NGS platforms include the 454 (Roche), Illumina, Solid and Ion Torrent, and PacBiosystems.

2.6.2. Pulsed Field Gel Electrophoresis (PFGE)

The pulsed field Gel Electrophoresis (PFGE) method is a laboratory technique that is widely used to produce a fingerprint for bacterial isolates. Usually, these isolates are related to a group of the same type of bacteria. In this method, the total DNA is digested by a restriction enzyme with rare cutting sites that recognizes and cleaves specific double-stranded DNA to generate a small number of large DNA fragments with high molecular weight (Chifiriuc et al., 2017). The high molecular weight DNA fragments favor migration and separation in the PFGE. The results then are analyzed and compared with known patterns. The advantages of using the PFGE technique comprised of high concordance with epidemiological related, high reproducibility, stable, high efficiency, and low cost (Chifiriuc et al., 2017). This procedure can be used for many bacteria, and for each bacterial strain, it is only need to change the restriction enzyme and electrophoresis conditions. However, there are some disadvantages associated with this method including: laborious and time consuming, bands of the same size may not be related to the same part of the chromosome, change in one restriction site can result in more than one band change, and some strains cannot be typed by this method (CDC, 2017).

2.7. Airborne organisms in food processing plants

Airborne bacteria may be present in droplets as bioaerosols; these bioaerosols can be an important source of contamination of food, surface, and sensitive manufacturing operations. Therefore, monitoring the airborne microorganisms of the food facilities may help identify the

source and prevent contamination of production chain. A great amount of research regarding the airborne contamination of food manufacturing plants has been reported. Heldman and Hedrick (1971) reported the potential for airborne contamination in food plants; Kotula and Kinner (1964) reported airborne contamination in poultry plants. Other studies investigated the airborne contamination of dairy plants and reported the airborne organisms as an very important source of contamination (Kang and Frank, 1989 a,b,c; Ren & Frank, 1992 a,b; Ravva and Sarreal, 2011). Airborne contamination in pork processing has also been reported (Kotula and Emswiler-Rose, 1988). Others have investigated the potential for airborne contamination in meat plants during processing (Kotula and Emswiler-Rose, 1988; Takahashi et al, 1989; Rahkio and Korkeala, 1997; Burfood et al., 2006; Schmidt et al., 2012), and most of their obtained results have indicated that airborne organisms are a potential source of contamination in meat products. In their investigation of the meat establishment plant, Sofos et al., (1999a) reported that contamination of meat can occur at various points during the slaughter process, cold storage, and processing of meats. Thus, the air is one of the potential sources of contamination of meats by both pathogenic and spoilage organisms (Kotula and Emswiler-Rose, 1988; Kang and Frank, 1989 a; Rahkio and Korkeala, 1997; Hadley et al., 1997). During slaughter and processing, aerosols are generated from dehiding of animals, carcass washing, equipment, workers' activities and air circulation. The aerosol particles may harbor pathogenic organisms and contaminate the products during slaughter and processing and could be an important source of contamination of meat and meat products. (Kotula and Emswiler-Rose, 1988; Burfoot et al., 2006). Rahkio and Korkeala (1997) investigated the carcass contamination by airborne organisms, and they found a strong association between carcass and airborne contamination. These authors concluded that air was an important source of contamination in slaughterhouse. *Salmonella* and *E. coli* O157:H7

are recognized as potential airborne organisms due to the presence of these bacteria in gastrointestinal tract of animals. These pathogens are shed in feces and may contaminate the environment and animal hide; and later when animals enter the slaughterhouse, the pathogens may become aerosolized during dehiding and splitting process. Also the animal intestines may come into contact with the carcass during slaughter processing and contaminate the carcass. The hide or skin of slaughtered animals has been recognized as a source of airborne organisms in slaughterhouses, especially for *Salmonella* and *E. coli* O157:H7 (Nottingham et al., 1974; Barkocy-Gallagher et al., 2003; Brichta-Hartay et al., 2008; Nou et al., 2003). Therefore, is it not surprising that the largest amount of contamination with airborne organisms has been reported to occur at dehiding and carcass splitting areas (Prendergast et al., 2004; Schmidt et al., 2012). However, the presence of these airborne pathogens in areas has a great potential to contaminate clean areas such as fabrication and chilling rooms (Schmidt et al., 2012). The prevalence of the airborne organisms in slaughterhouses varies depending on the processing area, the size and design of slaughterhouses (Prendergast et al., 2004). Therefore, monitoring and improving the air quality of food processing plants is important to reduce or prevent airborne contamination (Cundith et al., 2002). Many research have been conducted in meat processing plants to reduce microbiological contamination of carcasses and contact surfaces using different strategies and techniques. Washing of the carcass, organic acid treatments, trimming, and combination of these treatments were used to control contamination of carcass and contact surfaces (Cabedo et al., 1996; Anderson et al., 1987; Prasai et al., 1995; Dorsa et al., 1998; Cutter and Siragusa, 1994; Delmore et al., 1997; Castiilo et al., 1988). However, some of these methods that are used for controlling carcass contamination or surfaces also may contribute to generating of bioaerosols. Therefore, methods or interventions to control airborne organisms along with previous methods

are necessary. Currently, studies have been done to control airborne organisms in meat plants. Numerous technologies are available for this purpose. Patel and Nou (2008) investigated the effectiveness of a reactive oxygen species-generating system to control airborne organisms in meat processing plants, and they found this treatment able to reduce airborne organisms significantly. Other technologies that are used to control airborne organisms include the use of UV light and filtration along with electrostatic precipitation to capture airborne particles (Gardner and Shama, 2000; Georges and Feddes, 1995). Another method for controlling airborne organisms is using a germicidal air cleaning console unit in combination with a duct mounted air-cleaning unit (Cundith et al., 2002). These methods are found to be more practical to control airborne organisms in small meat-processing plants. Finally, although different technologies and strategies are available to control and capture airborne microorganism, improvement of sanitation program in food processing facilities is a key to success in reducing or eliminating the risk of airborne contamination.

2.7.1. Airborne organisms and factors affecting their presence

Microorganisms cause foodborne illness at various environmental conditions and in foods; those include bacteria, fungi, and viruses. Contamination can occur by different routes of transmission; it can be through person-to-person or environmental sources. Environmental matrices such as water, soil, surfaces and air are important reservoirs of microorganisms (Napoli et al., 2012). Air can play a major role as a reservoir for microorganisms in controlled and non-controlled environmental conditions. Airborne organisms can transmit from a source to a person or food products through aerosols, resulting in infection of the person and contamination of food products (Li et al., 2007; Burfoot et al., 2006).

Aerosols are defined as a suspension of fine solid particles or liquid droplet in the air or another gas, with size ranging from 0.001 to 100 μm (Hinds, 1982). The droplet nucleus consists of the airborne residue of potentially infectious aerosol from which most of the liquid has evaporated (Wells, 1934). In fact, airborne particles are a major cause of respiratory ailments in humans, causing allergies, asthma, and pathogenic infections of the respiratory tract (Li et al., 2007).

Thus, environmental exposure such as air is a common hazard for all such organisms (Tang, 2009). One of the most common sources of aerosols transmission is the ventilation of buildings. Li et al. (2007) investigated the relationship between the ventilation system, the airflow pattern and the spread of airborne infectious diseases, and they found a strong and sufficient evidence of the association between ventilation and the control of airflow directions in plants and the transmission and spread of infectious illness.

Therefore, airborne monitoring is necessary to measure air quality and identify critical situations (Napoli et al., 2012). The presence of airborne organisms depends on different environmental factors. Airborne organisms pass through various stages during transmission from a source to a secondary host to cause infection and usually require sufficient numbers of viable organisms to cause infection. Factors such as temperature, humidity (both relative and absolute), sunlight (ultraviolet light) exposure and even atmospheric pollutants affect the survival and presence of airborne organisms (Tang, 2009). These factors affect various airborne organisms in different ways and degrees, and can all act to inactivate these organisms (Tang, 2009). Goodlow and Leonard (1961) studied the viability and infectivity of airborne organisms in experimental analysis and demonstrated that the viability of these organisms is dependent on the various environmental and experimental factors. Therefore, the effects of each of these factors should be

considered to enhance aerosol/ airborne pathogens control in different environmental conditions. In addition, the process of aerosolization and impingement collection can physically damage the bacterial cell walls (Lundholm, 1982). Therefore, the use of an effective sampler to collect air samples is essential for detection of airborne organisms. Furthermore, conventional culture methods to count the number of airborne and viable organisms are not always useful because most of the bacteria are in a state of viable but not culturable or not able to form colonies after aerosolization (Heidelberg et al. 1997). Thus, cultural methods always have to be followed by modern automated rapid methods.

2.7.2. Methods of air sampling

Concern about the presence of airborne organisms and transmission of these organisms through bioaerosols is growing (Lundholm, 1982). As awareness of the airborne transmission is increasing, the demand for the best methods for measuring the number of airborne organisms by researchers and industries parties is also growing (Lundholm, 1982). Methods that are used for monitoring airborne organisms include the use of sedimentation plates, liquid impingers, slit and sieve impactors, filters, centrifugal samplers, filter system, and particles samplers (Kang and Frang, 1989a; Cvjetanović, 1958; Wirtanen et al., 2002). However, each of these air samplers works differently, and the principle of their performance may affect sampling efficiency for different species of bacteria (Lundholm, 1982). In addition, microorganisms are often injured due to the stresses of the aerosolized state and may result in failure detection of airborne organisms. Therefore, the type and suitability of the air sampler are important factors in monitoring the presence of specific microorganisms in the air of different food processing facilities. Air sampling using impingement and filtration, which subjects the organisms to

additional stress, may cause more injury that prevents their growth on non-selective media (Kang and Frang, 1989a). Lundholm (1982) compared and evaluated the efficiency and suitability of some commercially available air samplers, and he found that the Andersen sampler gave the highest viable bacterial counts in all environmental tested. While slit sampler and impinge gave lower counts than the Andersen sampler in all environments tested. Ren and Frank (1992) obtained the same results for Anderson sampler when they studied the airborne contamination in two commercial ice cream plants using different sampling methods. Therefore, selecting an adequate air sampler is an essential issue for successful detecting and monitoring of bioaerosols in food manufacturing plants. In spite of the availability of different types of air samplers and procedures to collect air samples, the ability of sampler or method to collect a large and sufficient volume of air is still needed for detecting and identification of airborne organisms (Kang and Frank, 1989; Radosevich et al., 2002).

2.7.3. Active and passive air sampling methods

In an active air sampling methods, an air sampler is used to collect a known volume of air in a liquid or a solid culture media or a nitrocellulose membrane depending on the type of the air sampler. Then, the collected air sample is analyzed using conventional culture methods or automated rapid methods. This system is usually used when the concentration of microorganisms in the environment is low and collection of the larger amount of air allows for the determination of airborne organisms (Napoli et al., 2012). While in passive sampling methods, the settle plates are used to collect the air sample. These sampling methods consist of standard Petri dishes containing non-selective or selective culture media; these plates are exposed to the air for a certain amount of time to collect air particles and incubated, which is also called sedimentation

methods. After incubation, the growing colonies are counted in CFU/plate/min or in CFU/hour (Kang and Frank, 1989; Pasquarella et al., 2000). Compared to active methods, passive methods are easy to perform and relatively inexpensive. In addition, permit the collection air particles in their original state. However, passive methods are more appropriate when the concentration of microorganisms is high, as at low concentration, the plates may not able to collect airborne organisms. Meanwhile, passive methods are subjected to drying and affected by air movement (Fang and Frank, 1989). Comparison between these two methods of air sampling is not easy as the concentration of microorganisms is not evenly distributed, and air sampling is performed in different areas with different kinds of airflow, different numbers of workers. Thus, with the presence of all these factors, which affect the final results obtained by either method, it is difficult to assess or compare their effectiveness (Andon, 2006; Pasquarella et al., 2000; Pasquarella et al., 2008). Many studies have been conducted to determine airborne contamination using different air sampling methods. Napoli et al. (2012) evaluated microbial contamination at operating theatres in hospitals and compared the effectiveness of active and passive methods. They demonstrated that both active and passive sampling methods were correlated when an exact protocol is used and concluded that both methods can be used for general monitoring of air contamination. This was contrary to other studies, which indicated that active sampling methods were better than settle plates (Verhoeff et al., 1990; Asefa et al., 2009).

2.7.4. Wetted Wall Cyclone air samplers

The concentration of microorganisms in the air is usually very small (Errington and Powell, 1969. Therefore, using an effective air sampler that works at a high flow rate and capable of concentrating a large volume of aerosol particles into a small volume in a liquid is an

essential issue for microbial analysis (Errington and Powell, 1969; Hubbard et al., 2011). Using small liquid volumes reduces the quantities of reagents, thereby minimizing the cost, and increasing the speed of analyses (Hubbard et al., 2011). The Wetted Wall Cyclone (WWC) is an air sampler that is used to collect bioaerosol particles in real-time from a single stage in a liquid. The WWC is designed with a complex multiphase flow to deliver very small liquid effluent flow rate of highly-concentrated aerosols (McFarland et al., 2010; Hubbard et al., 2013). The WWC is designed to have gravity fed water film along the inner walls of the cyclone, which allows the conversion of air samples into dilute bioaerosols in a liquid (Ahuja, 2010; Hubbard et al., 2013; Hu and McFarland 2007; McFarland et al., 2010). The liquid samples then can be analyzed with different detection methods. Simply the WWC has components that consist of an inlet, pre-separator, concentrator, aerosol-to-hydrosol transfer stage, and analyzer; all these parts together make the WWC a unique and effective air sampler. Studies have been conducted to isolate bioaerosol particles into a liquid using the WWC air sampler (King and McFarland, 2012; McFarland et al., 2010). The concentrated air sample is converted from the aerosol to the diluted liquid state to enable the analyzer to analyze the bioaerosols. Currently, three types of WWC collectors are available at Texas A&M University; the Aerosol Technology Laboratory with airflow rates of 100, 400, and 1250 L/min. Each of the WWC is equipped with diaphragm liquid scavenging pumps that allow a liquid flow rate of 0.275 L/min. But pumps were modified to use peristaltic pumps, which operate at flow rates of 3.4 ml/min (King and McFarland, 2012). The input liquid for collecting air samples is usually distilled water, but sometimes the addition of some other agents, such as a surfactant, is required to help in biological particle transport (King and McFarland, 2012). The amount of output liquid is dependent on environmental conditions and the WWC size and airflow rate. Thus, the amount of liquid sample output by the collector is

an important factor to increase the concentration of the sample and minimize collection liquid and reagent consumption (Hubbard et al., 2011; Hubbard et al., 2013).

2.7.5. Air samplers: wetted versus dry (sedimentation) systems

Sedimentation plates or settle plates are classical air sampling methods that have been widely used in the food industry (Kang and Frank, 1989a). Petri dishes containing non-selective media such as Tryptic Soy Agar (TSA; Difco, Sparks, MD), or selective media depending on the target organisms are used in settle plates methods. The plates are exposed to air for a given period of time and then incubated to grow visible colonies (Pasuarella et al., 2000; Napoli et al., 2012). The sedimentation plate's methods include the collection of a low volume of air sampling with low air flow rate for a short time. Plates are subjected to drying, which leads to stress to the targeted microorganisms. In addition, these methods of air sampling usually collect larger air particles and sensitive to air movement as it leads to deposition of the particles, consequently the distribution of particle-size may indicate a larger number of large particles (Griffiths and DeCosemo, 1994). These methods cannot be used for quantitating purposes, and at high bioaerosol concentration, the plates are non-countable (Holah et al., 1995). In comparison, the WWC provides advantages over the settle plates. The WWC provides real-time sampling, which collects aerosol particles in a liquid form (Hubbard et al., 2011; Hubbard et al., 2013). The liquid form of collected air samples allows analysis with advanced detection methods such as RT-PCR, which permits for quantitatively detection of airborne organisms (McFarland et al., 2010). In addition, its ability to collect air sample for long periods results in highly concentrated samples at a relatively high flow rate. The concentrated air samples give more opportunity of detecting airborne pathogens in real-time with consumption of a small amount of liquid (McFarland et al.,

2010). The ability of the WWC to sample for a longer time and the design and functionality of WWC systems make them unique air samplers to detect airborne organisms in food processing plants and other environmental conditions and more efficiently compared to other air samplers.

CHAPTER III

MATERIALS AND METHODS

3.1. Materials and Methods

3.1.1. Preliminary experiments

Before starting the research and collecting air samples, preliminary tests were conducted in the Aerosol Technology Laboratory to determine the stability of *Salmonella* in the sampling fluid during storage for extended periods. A greater stability would validate the adequacy for samples during sampling for an entire day, and also would ensure that the samples would remain stable even if they needed to be shipped overnight or for longer times. A cocktail of mid-log phase *Salmonella* strains including *S.* Agona, *S.* Typhimurium, *S.* Saintpaul, *S.* Heidelberg was prepared by individually reviving the cryopellets of organisms into 9 ml Tryptic Soy Broth (TSB; Difco, Sparks, MD) and incubated overnight at 37 ºC. Subsequently, each strain was aseptically streaked onto TSA plates and incubated aerobically for 18-24 h at 35 ºC. Then a number of grown colonies were inoculated into 9 ml TSB and incubated for 6 h to obtain the fresh culture. The culture then was centrifuged, the pellet resuspended in 10% PBS, then nebulized using a 6-jet Collison nebulizer (BGI, Waltham, MA) and captured by the WWC unit in Milli-Q water (Millipore ultrapure water) as collection liquid or Phosphate Buffered Saline (PBS) to determine if pure water might result in osmotic shock to the bacteria, resulting in lysis or loss of the captured salmonellae. For each suspension medium (water and PBS) the nebulized *Salmonella* cocktail was captured using the WWC and the suspension stored at room temperature (21 ºC) or under refrigeration (4 °C) for 7-10 days. The non-nebulized cocktail suspended in

TSB was used as a control. At regular intervals over 10 days at each storage temperature, a portion of the suspension was collected and diluted for plate count. The dilutions were plated following the Thin Agar Layer method (TAL) of Kang and Fung (2000) as a stress recovery method. This method consisted of selective media (25 ml agar added to a height of 6 mm), and then 14 ml of non-selective melted TSA (44-48 ºC) is added in two steps (7 ml/, then other 7 ml) to solidified selective media. Then the solidified overlaid plates were stored in a walk-in cooler until use. The sample then was plated onto a thin layer of XLT-4, overlaying with Tryptic Soy Agar (TSA) prior to continuing incubation up to 24 h (Kang and Fung, 2000). Typical colonies of *Salmonella* on overlaid XLT-4 (non-fermentative with a black center due to the production of H_2S) were counted and compared.

3.1.2. Beef harvesting establishments and sample collection

All samples were collected using WWC samplers, which have been described in Chapter II, Section 7.4. For this research, two small (establishments A and B) and two large (establishments C and D) establishments were sampled 3–4 times each. Establishment A and B were sampled during fall of 2016, and during spring, and summer of 2017. During the fall season, the enrichment time for collecting air samples was 18 h for plant A and B, while during spring and summer, the enrichment time was 18 and 36 h for all plants. For establishment C and D, each establishment was sampled twice; establishment C was sampled during spring and summer of 2017. While establishment D was sampled twice during summer of 2017. For establishment A and B, due to the size of these establishments, the slaughter stations were very close to each other, especially the stunning/exsanguination and the hide pulling areas, and the carcass splitting and carcass dressing areas. Therefore, two stationary WWC units were placed,

one at the initial steps of the harvesting process knocking/exsanguination area and hide pulling, and the other at the end of the carcass harvesting process between carcass splitting and final dressing before going to the chiller. While for each establishment C and D, one stationary WWC unit was placed in the dehiding area and the other was placed in the fabrication room as these areas need to be sampled for a long time (4 hours) morning and (4 hours) afternoon, the WWC units with smaller size and weight were used to change the place of sampling following the slaughtering process, the dynamic unit was also used to collect a sample from chiller room. The samplers were adjusted to provide an airflow during sampling of 100 L/min and were left for the entire working day, a total of 8 hours/day. Samples were also collected during the entire process and each long-term air sample represented an approximate total volume of 48,000 L. A sterile 30 x 115- mm Nalgene screw-cap centrifuge tube was attached to the WWC systems to collect the wet samples during the morning sampling. This tube was replaced at mid-day with a new tube for continued sample collection during the afternoon. Along with two stationary (WWC) units for the day-long sampling, two smaller (portable) air samplers, with the air flow rate set at 100 L/min were placed consecutively for 15 min at the dehiding, shank removal, evisceration, and carcass splitting areas to dynamically sample air at each stage. This enabled tracking of pathogens potentially aerosolized during the different processing stages. Duplicate air samples were taken at each sampling site for further comparison of testing methods. Upon completion of the sampling time, the PBS vial with the sample was removed and transported under refrigeration to the Aerosol Technology Laboratory at Texas A&M University for analysis. Transport time from the establishment and the laboratory was less than 2 h for plant A and B, while for plant C and D the transport time was 15-17 h due to the distance between these plants and Texas A&M University. When the sample needed to be stored overnight, storage was at 4

°C. Preliminary experiments indicated that the sample was stable for at least 7 days. Upon arrival in the Aerosol Technology Laboratory, one of the duplicate samples was taken to the Food Microbiology Laboratory. Samples from two shifts (morning and afternoon), and samples from each shift with similar areas were combined to obtain one sample for each specific area, then each sample was mixed with PBS to a volume of 25 ml, and then added to 25 ml of the appropriate enrichment broth. The time between sample collection and testing ranged between 3 and 18 h. When the sample needed to be stored overnight, storage was at 4 °C. Preliminary experiments indicated that the sample was stable for at least 7 days.

3.1.2.3. Microbiological analysis

Microbiological analysis of air samples included both quantitative and qualitative assays. Quantitative analysis consisted of direct plating onto selective and differential media to enable enumeration in case those pathogens were present in the air samples at concentrations sufficiently high for direct plate counting, whereas qualitative assay included detection of STEC and *Salmonella* utilizing an automated Crystal Diagnostic (CDx) system and RT-PCR. The direct plating CDx analyses were carried out in the Food Microbiology Laboratory and RT-PCR was carried out in the Aerosol Technology Laboratory.

3.1.3. Quantitative Analysis (Direct plate testing)

For quantitative analysis, 0.1 ml of PBS containing the air sample in suspension was directly plated onto a plate of mPossé agar (Posse et al., 2008) for STEC enumeration, and on XLT-4 for *Salmonella* enumeration. The (TAL) method of Kang and Fung (2000) described in Section 1.1 was used for the direct plating analysis on mPossé and XLT-4 agars to ensure

accurate enumeration in case of any potentially injured cells of STECs or *Salmonella* being present. After incubation, typical colonies (3 colonies/ plate) of STEC or *Salmonella* (on their respective selective media) were isolated and confirmed by biochemical and serological tests using API 20E strips (BioMérieux, France). Serotype O157:H7 was confirmed by slide agglutination test using anti-O157 and flagellar H7 antiserum (Difco, Sparks, MD), while other STEC were confirmed using gel-based PCR. To confirm the identity of *Salmonella*, biochemical tests were conducted on Triple Sugar Iron Agar (TSIA), Lysine Iron Agar (LIA), and Urea medium. Finally, serological confirmation was conducted by the slide agglutination test using agglutination with Poly a-z antiserum (Difco, Sparks, MD).

For confirmation of presumptive STEC isolates by gel-based PCR, the DNA first was extracted using a ZyppyTM Plasmid Miniprep Kit (Zymo Research). The DNA extraction kits were used according to the manufacturer's instruction. The extraction was performed by adding 600 µl of bacterial culture grown in Tryptic Soy Broth (TSB) to a 1.5 ml micro-centrifuge tube. Then 100 μL of 7X Lysis Buffer added to the bacteria culture and mixed by inverting the tube 4-6 times, followed by incubation for 1-2 minutes. After mixing, the color was monitored for a change from opaque to clear blue, indicating complete lysis. After that, a 350 μL of cold Neutralization buffer was added and mixed thoroughly by inverting the tube up and down. After mixing, the color changed to yellow, indicating complete neutralization. The tubes then were centrifuged for 2-4 minutes at 11,000 x g. Afterward, a Zymo-Spin^{TM} IIN column was placed in a collection tube and the supernatant from the previous step was transferred into the Zymo-Spin^{TM} IIN column and was centrifuged for 15 seconds at 11,000 x g. The flow-through was discarded and Zymo-Spin^{TM} IIN was returned to the same collection tube. Next, 200 μL of Endo-Wash Buffer was added to the column and centrifugation for 30 seconds at 11,000 x g. After that, 400

μL of Zyppy TM Wash Buffer was added to the column and centrifuged for 1 minute at 11,000 x g. The column was then transferred into a clean 1.5 ml microcentrifuge tube and 30 μL of Zyppy TM Elution Buffer was added directly to the column matrix, followed by incubation for one minute at room temperature. Finally, to elute the DNA, the tube was centrifuged at 11,000 x g. The extracted DNA was then stored at -20 °C until used.

For processing large numbers of samples, the lysis and neutralization steps should be performed in groups of less than 10 preps to avoid excessive lysis, which can result in denatured DNA. After DNA extraction, the DNA concentrations were determined using NanoDrop® ND-1000 spectrophotometer (Saveen and Werner). The concentration was measured in ng/µl. Then a conventional PCR method was performed using MyCyclerTM Thermal Cycler (Bio-Rad) at Department of Nutrition and Food Science. The PCR reaction was set up following the guidelines of Liang and Johnson (1988) and Sambrook et al. (1989). Each PCR mixture consisted of 25 μL total, which contained the DNA template (5 μL), the forward and reverse primers for *stx* and *eae* genes (100 mM, 1 μL each) (Table 1), 12.5 μL master mixed (Promega Corp.,Madison, WI), 10× PCR buffer (5.5 μL, Promega). The *stx* primers Forward and Reverse (Integrated DNA Technologies, Inc., Coralville, IA) were used to amplify the DNA fragment size (132 bp). The amplification conditions were 95 °C initial denaturing step for 10 min, 35 cycles of 95 °C for 15 s, 60 °C for 1 min annealing, and 72 °C for 30 s/kb extension, a final extension at 72 °C for 10 min and a cooling step at 40 °C for 30 s. The PCR products were analyzed with gel electrophoresis, using 2% agarose gel in 1× TAE (Tris-acetate-EDTA) buffer at 100 V. The bands in different lanes were compared by staining with ethidium bromide and visualized with UV light and photographed with the ChemiDocTM Touch (Imaging system).

Table 1. *Primer information for conventional PCR in this study*

Primer	Sequence	Amplicon size	Reference
Stx F	5’ TTT GTY ACT GTS ACA GCW GAA GCY TTA CG 3’	132	Integrated DNA Technologies
Stx R	5’ CCC CAG TTC ARW GTR AGR TCM ACD TC 3’		
Eae F	5’ CAT TGA TCA GGA TTT TTC TGG TGA TA 3’	102	Integrated DNA Technologies
Eae R	5’ CTC ATG CGG AAA TAG CCG TTM 3’		

3.1.4. Qualitative detection of STEC and *Salmonella*

3.1.4.1. Detection of STEC and Salmonella using Crystal Diagnostic (CDx)

All direct plating and qualitative detection of pathogens were conducted in the Food Microbiology Laboratory of the Department of Animal Sciences at Kleberg Center. Qualitative detection of STEC and *Salmonella* was conducted using the CDx Pathogen Detection System (Crystal Diagnostics, Denver CO), a commercially available liquid crystal-based immunoassay pathogen detection system. Each sample designated for pathogen testing was divided into two portions, each portion included approximately a volume of 2.4 m^3 of the air sample, and then each portion was mixed with PBS to a volume of 25 ml, and then added to 25 ml of the appropriate enrichment broth. For STEC, the 25-ml sample was added to 25-ml of 2x modified Tryptic Soy Broth (mTSB) + novobiocin supplemented with VCC (Vancomycin+

Cefixime+Cefsoludin) pre-warmed to 40 °C, and incubated for 18 hours at 42 °C. For *Salmonella*, the 25-ml sample was added to 25-ml of 2x Universal Pre-Enrichment Broth (UPB) and incubated for 18 hours at 42 °C. For both pathogens, the enriched sample after incubation was gently mixed by hand and a portion of 30 ml of the corresponding enriched medium was transferred into labeled conical tube allowing the sample to settle for about 10 minutes.

The incubation time for the enrichment step in CDx analysis was later adjusted based on an experiment to determine the need for extending the enrichment time to increase recovery of the pathogens. Aerosolization of *Salmonella* from beef hide was simulated by shaking a piece of beef hide for approximately 15 min in a biosafety cabinet. Non-inoculated hide pieces were used to produce naturally occurring *Salmonella*, while a positive control consisted of inoculating hide pieces with *Salmonella* ser. Typhimurium. After simulated aerosolization, the air in the cabinet was collected in settle plates (Petri dish) and growth from these plates was directly transferred to UPB and incubated followed by incubation for 24 h. At 2 h intervals, samples were collected from the enrichment broths and plated on selective and differential XLT4 medium to build a growth curve of *Salmonella* naturally present in the sampled air from hide shaking, and in the positive control. For each air sample, CDx analysis was conducted as described below. Growth curve data were fitted in Excel and for each growth curve, the maximum growth (amount of generations) and generation time were calculated based on the following equations.

$2^{\text{number of generations}}$ x initial number of bacteria = total No. of bacteria present after n generation

Number of generation = (log cells at end of the selected time period during exponential phase) - (log cells at beginning of selected time period during exponential phase)/0.301

Generation time = 60 min x hours/ number of generations

Lag time was calculated by modeling a theoretical curve with no lag, based on the generation time and the initial *Salmonella* concentration, then determining the time at which a specific count was reached during the exponential phase for both the theoretical and the actual curves. The difference between these two time values is the lag time. These curves were conducted in 3 trials, each including 3 replicates. (N=9).

Before preparing samples for analysis, the CDx Reader and incubator (Thermo Scientific Heratherm, Waltham, MA) were turned on, setting the incubator at 17 °C. All CDx materials and bio- cassettes were pre-warmed in a Mini Controlled Environment, which is a component of the CDx testing equipment. The lyophilized CDx Liquid Crystal (LC) was rehydrated with CDx LC Buffer according to the package instructions and was heated to 60 °C in a heat block for approximately 20 min. After 20 min, the hydrated LC was vortexed briefly and transferred to the 40 °C heat block, and was remaining there until ready for use.

The CDx method involves the use of immunomagnetic separation. Microspheres coated with the appropriate antibody (Crystal Diagnostic, Ltd, CO) were prepared from the testing kit for each pathogen. For STEC analysis, three antibody kits are available (Negative Control, Panel A, and Panel B vials). For *Salmonella* and for *E. coli* O157, two antibody kits are available (Negative Control and Panel A vials). Each vial was mixed using a vortex mixer for 10 s and, for each test sample, 195 µL was transferred to a separate well on a deep well microplate (3 or 2 wells are needed for each sample depending on the test type). Then, 1.4 ml of sample enrichment was transferred to a 1.5 ml microcentrifuge tube, to spin the samples on a mini-centrifuge (VWR MiniFuge set at 2000 x *g*) for 80 s to pellet sample debris. After centrifugation, 300 µL of sample supernatant was transferred to the corresponding Negative Control, Panel A, and Panel B wells in the deep-well microplate. After sample addition, the deep-well plate was placed on a

microplate vortex mixer at low speed to ensure no spillover between wells and mixed for 10 minutes. After mixing, the immunomagnetic separation was carried out. The deep well microplate was removed and placed on the magnetic separation block for 3 minutes. The supernatant was removed carefully and discarded using an 8-channel multipipette without disturbing the microsphere pellet. Then, while keeping the microplate on the magnetic base, 1 ml of PBST was added to each well containing microspheres. The microspheres were allowed to pellet on the magnet for 2 minutes. After 2 minutes on the magnet, the entire volume of supernatant was removed and discarded. Then the microplate was removed from the magnetic base and the beads were re-suspended in 25 μL of PBST. Then the deep-well plate was placed back on the vortex at low speed to maintain bead dispersion. For the CDx test, the prepared CDx LC was mixed for 3 seconds on low speed, and then an aliquot of 90 μL of CDx LC was transferred into 200 μL tubes for each sample prior to transferring 18 μL of the appropriate microspheres to an LC aliquot. The microspheres were gently mixed to be run in the first cassette; using a pipette or a vortex mixer at low speed. After mixing, 50 μL from each mixture was loaded into corresponding labeled inlet ports of the BioCassette. Once loaded an appropriate pipette with tip was used to add samples into the chamber until filled. Then the BioCassette was inserted in the CD x Reader. The result is displayed in the system after the tests are complete as positive, negative, or retest for each sample. The remaining microspheres in the pellet were reserved for further confirmation in case of a positive test.

After results of CDx analysis were obtained, which usually take about 4 hours including sample preparations, the positive results were confirmed by mixing the remaining of the pellet containing the microspheres in the microplates, with 100 μL peptone water. Then, a loopful of this microsphere suspension was streaked onto mPossé agar for STEC-positive samples or onto

XLT4 agar for *Salmonella*-positive samples. All plates then were incubated at 35 °C for 24 h. After incubation, plates were examined for the development of typical colonies on the corresponding selective and differential medium. In case those typical colonies had grown on the selective and differential media, further confirmation was performed by taking three colonies from each plate and conducting biochemical and serological tests, and conventional PCR for non-O157 STEC isolates, as described in subsection 1.3.

3.1.4.2. Detection of STEC and Salmonella using real-time PCR (RT-PCR)

All DNA extraction and RT-PCR testing were conducted at the Aerosol Technology Laboratory of the Department of Mechanical Engineering in the ENPH Engineering Physics Building. DNA was extracted using alkaline lysis. This method is a modification of the method described by Zhou et al., (1990). In this method, the collected sample was centrifuged for 5 min, and then added with 300 µl TENS (0.1 M NaOH, 10 ml 10X TE, and 2.5 ml 20% SDS) and mixed gently using vortex mixer at low speed for 20 sec. The tubes then were incubated for 10 min at room temperature. After 10 minutes, the sample was placed in a beaker containing an ice slurry and then 150 µl 3N sodium acetate was added to precipitate the protein. Next, the sample was mixed briefly with a vortex mixer and centrifuged for 2 min at 13,000 × *g* at 17°C. The supernatant was transferred to sterile 1.5 ml microcentrifuge tube and then 10 µl of Poly Acryl Carrier (PAC, MRI, Waltham, MA) was added to each tube, inverting gently before adding 1 ml of 100% isopropanol, inverting gently 10 times until well mixed. The tubes were centrifuged for 20 min, the isopropanol was removed carefully without disturbing the DNA pellet and then the DNA was washed with 1 ml ice-cold 100% ethanol and vortexed until the pellet was separated from the bottom of the tube. The tubes then were centrifuged for 10 min, and the supernatant was

carefully removed without disturbing the DNA pellet. Finally, the pellet was air dried and dissolved in 50 µl of DNA hydration (sterile DNA-free MQ water) solution. The DNA concentration was determined by measuring optic density (OD260) using a spectrophotometer (NanoDrop Technology, Wilmington, ED).

Although RT-PCR was used, only the qualitative data was relevant to this study. The quantitative was part of another study conducted at the Aerosol Technology Laboratory at Texas A&M University.

To prepare for RT-PCR analysis, the extracted DNA was added to PCR tubes/wells containing PCR assay reagents, and amplified in an automated thermocycler/ analyzer. However, for the spring and summer samples whole cell qPCR method was used adding 3 µL of the collected bioaerosol samples directly to the PCR reactions to result in genomic copy number (GCN) per m^3 of collected air. For standard curve fresh mid-log phase bacteria with known CFU/mL concentration were used, assuming that the culturable counts are equal to the actual cell counts. RT-PCR was conducted in a volume of 10 µl in a 96-well plate. A sample for RT-PCR analysis was prepared by a mixture containing 5 µl of 2x Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 1 µl Forward Primer 1 µl Reverse Primer (sequences for *stx* and *eae* variants were used for STEC, the sequences of *invA* gene for *Salmonella*, and 16S rDNA primers were selected for 16S rRNA), and 3 µl of template DNA. All reactions included a negative DNA control without template and a positive control containing strain specific DNA.

Using AB StepOne RT-PCR System (AB, Foster City, CA), the RT-PCR conditions were optimized and set as follows: heat at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 60 s. Fluorescence signals were measured once in each cycle at end of the extension step. After PCR amplification, melting temperature (Tm) curve

analysis was performed to verify the presence of the amplicon with the correct size. The PCR products were cooled to 65 °C and then slowly heated to 95 °C at a rate of 0.2 °C/s. Fluorescence signals obtained were used for continuously monitoring to confirm amplification specificity (Wong et al., 2005; Van Guilder et al., 2008).

The DNA of the positive samples from establishments A and B were sent to the Institute for Cellular and Molecular Biology at the University of Texas at Austin for DNA sequencing using a 16S rRNA-based technology, using Illumina Next-Generation sequencing. For both STEC and *Salmonella*, their evolution can be characterized using Illumina Next-Generation Sequencing Technology, which is based on the 16S rDNA sequencing of the bacterial chromosome. The ability of Next-Generation Sequencing to produce a large volume of data in a short period of time makes it possible to sequence and identify multiple bacterial strains at a time by comparing their 16S rRNA gene. By this method, it is possible to characterize microbiomes from samples, which are difficult to study otherwise (Clarridge, 2004; Mayo et al., 2014). The steps of the Next-Generation Sequencing include purification of extracted DNA, amplification of 16S rRNA fragments, and by attaching adapters to the fragments, anchor them to chip surfaces for amplification (Clarridge, 2004; Mayo et al., 2014).

Table 2. *Sequence of oligonucleotides used for real-time PCR in the study*

Primer Name	Amplicon (bp)	Sequence
Stx F (Forward)	132	5’ TTT GTY ACT GTS ACA GCW GAA GCY TTA CG 3’
Stx R (Reverse)		5’ CCC CAG TTC ARW GTR AGR TCM ACD TC 3’
Eae F (Forward)	102	5’ CAT TGA TCA GGA TTT TTC TGG TGA TA 3’
Eae R (Reverse)		5’ CTC ATG CGG AAA TAG CCG TTM 3’
invA 139 F(Forward)	284	5’ GTGAAATTATCGCCACGTTCGGGCAA 3’ (287-312)
invA 141 R (Reverse)		5’ TCATCGCACCGTCAAAGGAACC 3’ (571-550)
16S 1369 F (Forward)	123	5’ CGG TGA ATA CGT TCY CGG3’
16S 1492 R (Reverse)		5’ GGT TAC CTT GTT ACG ACT T3’

The positive isolates (stains) from air sampling for both *Salmonella* and STEC were maintained on Tryptic Soy Agar (TSA) slants.

3.1.5. DNA characterization of isolates

` All STEC and *Salmonella* isolates were shipped to the Food Safety and Intervention Technology Research Lab of the USDA-Agriculture Research Service (FSITL) for DNA fingerprinting using PFGE. Prior to shipping to IRS, all *Salmonella* and STEC isolates were streaked onto TSA slants and stored in refrigeration (4-5 °C) until the time of shipping. Before

shipping, the slants were prepared according to Texas A&M Environmental Health and safety protocol for shipping and packing hazardous materials.

3.1.6. BAX PCR System confirmation

Prior to characterization, all isolates were confirmed for identity using a BAX system (BAX System Q7, Hygiena, Wilmington, DE) at the FSITL. BAX is an automated system that uses RT-PCR technology. The BAX PCR system for screening *Salmonella* or STEC includes several steps, including sample preparation, cells lysis to release DNA for PCR, then transferring of lysates to PCR tubes, then loading PCR fragments into The BAX® System instrument, finally, reading the results. The system identifies a specific DNA fragment, unique to *Salmonella or* STEC. The positive isolates (stains) from air sampling for both *Salmonella* and STEC were maintained on TSA slants. All strains were grown in brain heart infusion agar (BD Biosciences) plates at 35 °C overnight and then went through BAX screening. The screening was done by transferring 0.05 ml from each overnight sample to lysis tubes containing a digestion buffer. Then samples were heated to 37 °C for 20 min and then heated at 95 °C for 10 min. The samples then were cooled down for 5 min in a cooling block. After that, 50 µl from each sample of the lysate was transferred to PCR tubes. The tubes then placed in BAX system cycler, and run based on the manufacturer's protocol (Qualicon, D. 2003; Bailey and Cosby, 2003).

3.1.7. Pulsed field Gel Electrophoresis (PFGE)

The PFGE analysis was performed in the USDA-ARS Food safety and intervention Technology Research laboratory following the standard operating procedure of the Centers for Disease Control and Prevention CDC (2013). Prior to PFGE analysis, all *Salmonella* and STEC

isolates were streaked individually onto TSA supplemented with 5% defibrinated sheep blood (TSA-SB) plates for confluent growth. The plates were then incubated at 37 °C for 14-18 hours. After the incubation period, plugs were made according to standard procedure (CDC, 2013a). TE buffer was prepared by mixing 10 ml of 1 M Tris (pH 8.0), 2 ml of 0.5 m EDTA pH 8.0, and diluted to 1000 ml with sterile Ultrapure Clinical Laboratory Reagent water (CLRW). One percent plug agarose was prepared in the TE buffer. The SeaKem Gold (SKG) agarose (0.50 g) was weighed in and placed into 250 ml screw-cap flask, and then 50 ml of prepared TE buffer is added and swirled gently until the agarose is dispersed. After that, the flask with dispersed agarose was microwaved for 30 s and then gently mixed for 10 s. The flask with agarose was placed in water bath at 55-60 °C for 15 min to equilibrate the agarose. In this procedure, SeaKem Gold agarose was used for PFGE plugs because it is proved that it provides added strength to the plugs minimizing breakage of plugs during the lysis and washing steps. After that, the cell suspension buffer (CSB) was prepared by adding 100 ml of 1M Tris (pH 8.0), with 200 ml of 0.5 M EDTA (pH 8.0), and diluted to 1000 ml with CLRW. Then 2 ml from CSB was transferred into small-labeled tubes with culture numbers (12mm x 75 mm Falcon tubes). Then the growth from agar plate was removed using cotton swab sterile with CSB and the cell suspended in CSB by spinning the swab cotton gently. The concentration was adjusted by diluting with sterile CSB or by adding additional cells. PFGE plugs molds wells were labeled with culture number. Then 400 µl adjusted cell suspensions were transferred to labeled 1.5 ml microcentrifuge tubes. Then 20 µl of proteinase K (20 mg/ml stock) was added to each tube and mixed gently with a pipet tip. Then 400 µl melted 1% SeaKem Gold agarose was added to 400 µl cell suspension; mixed gently pipetting mixture up and down for a few times. The temperature was maintained for melted agarose by keeping a flask in a beaker of warm water (55-60 °C). After addition of the

agarose, immediately, part of the mixture was inserted in the wells of plug mold. For this analysis, two plugs were used for each sample. The plugs were left at room temperature for 15 minutes to solidify. The generation of cell suspension and the subsequent casting of the plugs should be performed as rapidly as possible in order to minimize premature cell lysis. The cells were lysed in plugs using two plugs of the same strain in the same 50 ml tube. Next, 50 ml polypropylene screw-cap is labeled with culture numbers and used for lysis of the cells. Cell lysis buffer (50 mM Tris: 50 nM EDTA (pH 8.0) and 1% Sarcosyl) was prepared by mixing a 50 ml of 1 M Tris, 100ml of 0.5 M EDTA, and 100 ml of 10% sarcosyl (N-Lauroylsarcosine, sodium salt) in labeled 50 ml polypropylene and diluted to 1000 ml with CLRW. For each tube, 5 ml cell lysis buffer is needed, and 25 µl of proteinase K stock solution (20 mg/ml) is needed. After calculating the required volumes of each cell lysis and proteinase K buffer, a master mix was prepared by measuring the correct volume of cell lysis buffer and proteinase K and placed into a test tube and mixed together. The final concentration of proteinase K in the lysis buffer was 0.1 mg/ml. Then 5 ml of the master mix was added to each labeled 50 ml tube. The plugs (6-mm wide spatula) were transferred from mold to labeled tube. The tubes were then placed in a rack and incubated in a 54-55 °C shaker water bath for 2 hours with agitation (150-175 rpm). The tubes were then removed from the water bath, and lysis buffer was discarded. The plugs were then washed with pre-heated (54-55 ºC) 15 ml of ULRW and shacked in a 54±1 ºC water bath for 12±2 minutes, the water then discard. The washing step was repeated one more time. Then the plugs were washed with (12±2 ºC) pre-heated (52±2 ºC) TE buffer (10 mM EDTA, pH 8.0) and shacked in water bath (52±2 ºC) for 12 minutes and repeated 4 times and the liquid was discarded after each washing time, and 5 ml sterile TE was added to each tube. Then, the DNA in agarose plugs was restricted using Xbal enzyme (CutSmart; New England Inc., Ipswich, MA).

This was achieved by preparing a master mix using 10X restriction buffer (20 µl) (Roche Applied Science) and diluted with 1:10 Ultrapure sterile water (180 µl) (CLRW). Then, a 200 µl from prepared master mix was added to labeled 1.5 ml microcentrifuge tubes, and the plug removed from TE and placed in sterile disposable Petri dish. A slice of 2.0 mm wide was cut from each test samples and the appropriate number of slices of the CDC standard (*Salmonella* ser. Braenderup H9812) with a single edge razor blade and transferred to tube with diluted restriction buffer. The samples and control plug slices were incubated in a 37 °C water bath for 10 min. After incubation, buffer was removed from plug slices using a pipet fitted with 200-250 µl tip. After removing liquid from slices plugs, 0.5 X Tris-Borate EDTA buffer (TBE) was made by mixing (200 ml) of TBE with 1800 ml of CLRW. Then 1% SeaKem Gold (SKG) agarose gel made as described previously. Restricted plug slices removed from water bath, and buffer was removed and 200 µl 0.5X TBE was added and incubated at room temperature for 5 min. The plug slices were removed from tubes; and loaded on comb teeth (lanes) 1, 5, 10, (10 well gel). Then samples were loaded on remaining teeth and noted locations. The plug slices then allowed to dry on the comb for 3-5 min. The agarose (cooled to 55-60 °C) was poured carefully into the gel form. Then 2 L freshly prepared 0.5 X TBE was added. 1% pulsed field agarose gel was run for 19 hours with initial and final switch times of 6. 76 s and 35.38 s, and low MW of 30 kb, high MW of 700 kb for *Salmonella* with Voltage 6V and included Angle of 120°, While for STEC, the initial and final switch times were 2.16 s, 63.8 s, respectively, with low MW of 50 kb and high MW of 400 kb, voltage 6 V, and included Angle of 120°. After electrophoresis was completed, gel was stained with ethidium bromide for 30 min in covered container. The gel was distained in 500 ml reagent grade water for 90 min; the water was changed every 20 min. Finally, the image of gel was taken using a Gel Doc 1000, 2000, EQ (CDC, 2013a).

3.1.8. Data Analysis

For count data, values were transformed into log value and then analyzed by ANOVA using the JMP Pro 13 statistics software. Positive and negative sample data for STEC and *Salmonella* were compared by (Chi-square) non-parametric statistical methods for binomial distributions and logistic regression to determine the effect of season, plant size, and processing area. Percentages of positive samples obtained by non-enrichment RT-PCR and immunoassay, which involved enrichment, were calculated from confirmed STEC and *Salmonella* positive samples and compared by the Chi-square test. Comparison of proportions of positive and negative samples by RT-PCR and by CDx, or by each of these methods alone, was conducted using two sample proportions analysis using JMP Pro 13 statistical software. The Illumina sequencing results were evaluated using the Qiime pipeline to delineate the microbiomes in the collected bioaerosol samples.

CHAPTER IV

RESULTS AND DISCUSSION

4.1. Preliminary experiment

To validate the applicability of the air sampling system for the collection of *Salmonella* serovars, the stability of *Salmonella* suspended in the air sample generated by nebulization in pure water (Milli-Q) was compared as a function of the temperature of storage and the plating method was used (Figure 1). When the samples were stored at refrigeration temperature (4 °C), the populations of *Salmonella* decreased at a constant rate during storage ($P < 0.05$), whereas at room temperature, counts increased significantly. Nevertheless, the stability of the *Salmonella* in the sample was determined not to be acceptable when using pure water for suspending the aerosol sample during the WWC sampling. No differences ($P > 0.05$) were observed in the counts that obtained by a direct plating on XLT4 when compared to the counts obtained by the TAL method of plating first on XLT4, incubating and then adding an overlay with TSA, which indicated that the reduction observed was represented true lethality and not a sublethal injury due to the osmotic stress in the ultrapure water (Milli-Q) (Kang and Frank, 1989). When the nebulization of the *Salmonella* cocktail was conducted in MIlli-Q water or in PBS, the survival of this pathogen was significantly improved when suspended in PBS.

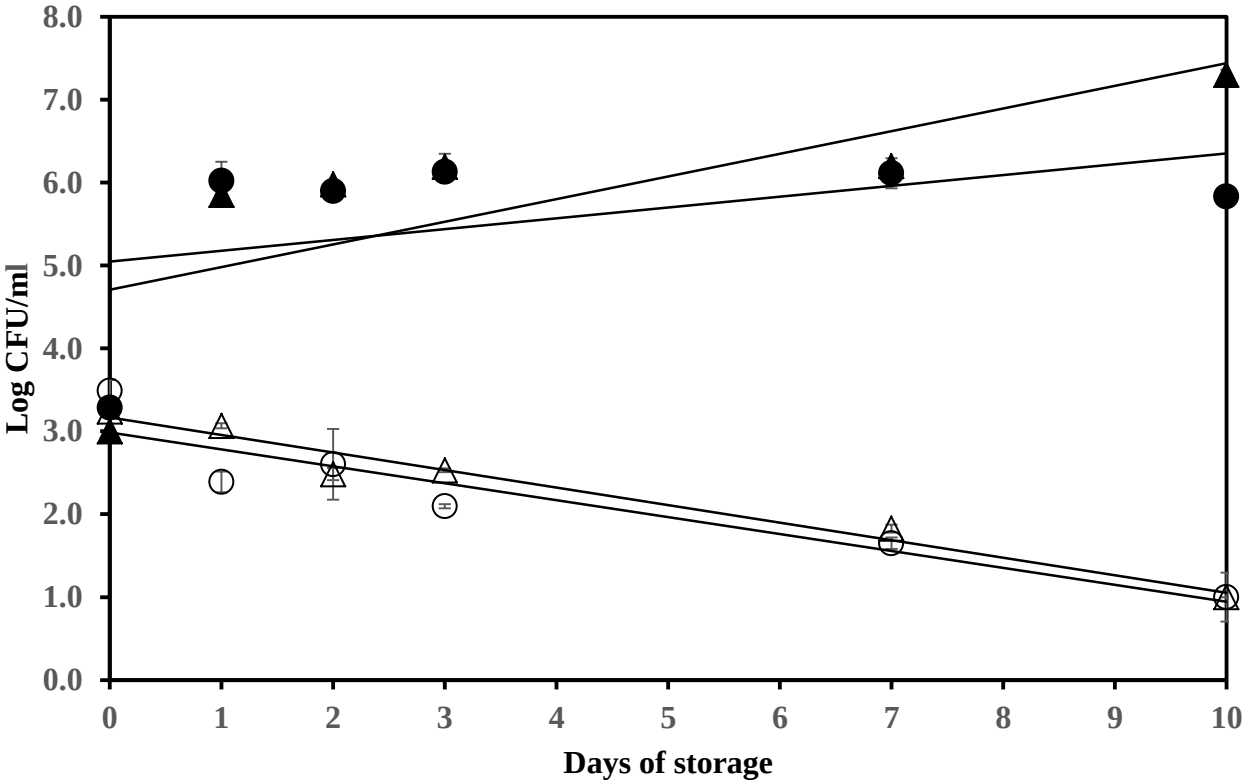

Figure 1. *Stability of* Salmonella *nebulized in Milli-Q water (ultrapure water) during storage at refrigeration (open symbols) or at room temperature (solid symbols). Counts were carried out by direct plating on XLT4 agar (circles) or by resuscitation method plating on XLT4 agar, incubating at 35 °C for 3 h and overlaying with tryptic soy agar and continuing incubating at 35 °C to complete 24 h (triangles).*

The data in (Figure 2) show the ratio of counts at each sampling time to the counts immediately after sampling (zero time). This ratio was calculated because the initial concentrations of *Salmonella* in PBS and pure water were different. This would have biased the data if the log counts had been compared. The ratio is smaller if there is a reduction in counts, and larger if there is growth. It was evident that when storing at (4 °C), the salmonellae suspended in pure water decreased during storage ($P < 0.05$), whereas the counts did not change over the 10-day storage period at 4 °C when *Salmonella* was suspended in PBS. As indicated before, an increase was observed for the samples suspended in pure water stored at room

temperature. No literature was found to explain the reason for this increase. As a result of these preliminary experiments, the sampling method using the WWC was established to be conducted by placing a container with sterile PBS to collect and suspend the air samples in the WWC, and to maintain the samples at refrigerated temperature until testing.

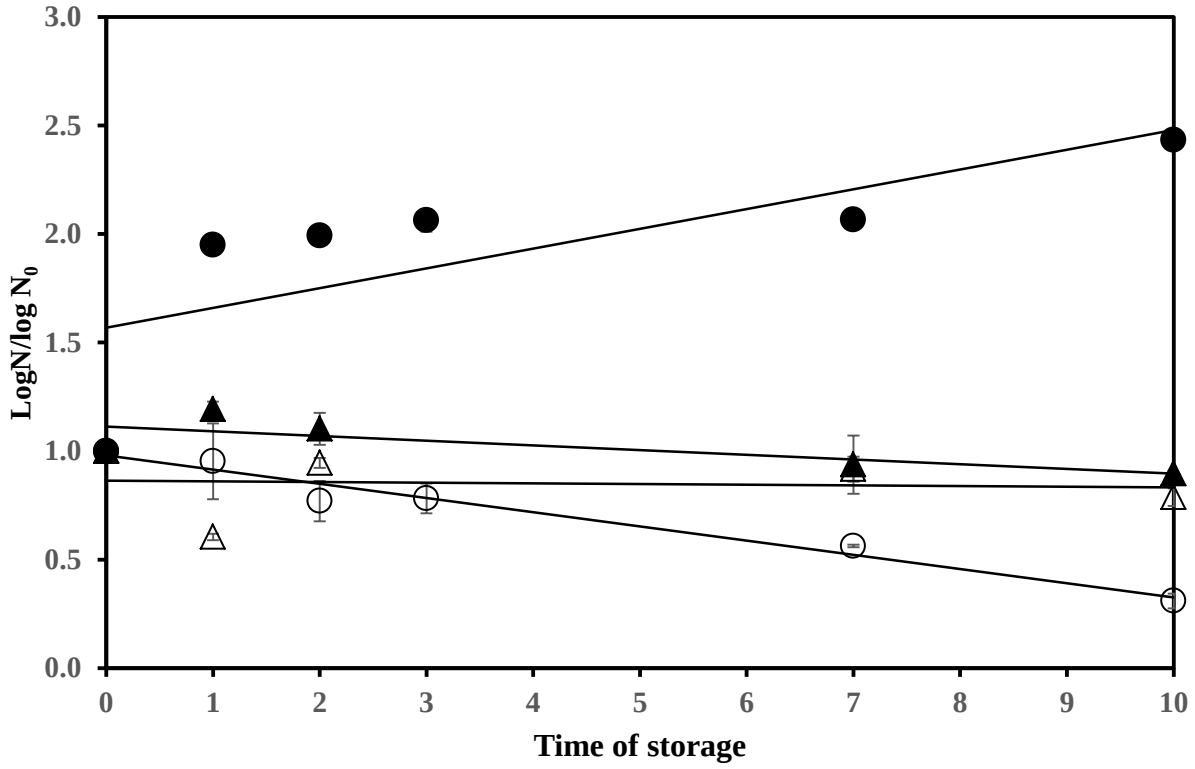

Figure 2. *Survival and growth of* Salmonella *suspended in Milli-Q (ultrapure) water (circles) or Phosphate buffered saline (PBS, triangles) during storage at 4 °C (open symbols) or room temperature (solid symbols). Log N/logN0 is the ratio of the log count at each sampling time divided by the log count at the beginning of storage (zero time).*

4.2. Detection of STEC and *Salmonella* by direct plating

During the first sampling at establishment A, a more intensive sampling was conducted to ensure that all methods were being followed adequately and to acquire confidence in the

procedures. Sampling was conducted on two consecutive days. Four WWC units were placed in the facility, two of these were portable, and moved every 15 min during sampling. Other 2 WWC units were stationary with a flow rate of 100 L/min and were used for the long-term sampling. For fall of 2016 sampling, this establishment was again sampled in the same manner as the first time. None of the samples collected during the two sampling days at establishment A tested positive for STEC or *Salmonella,* by a direct plating method. Similarly, samples collected from establishment B, and from the air in the feedlot that supplies cattle to establishment A, tested negative for STEC or for *Salmonella*, by a direct plating method (Table 3).

For spring and summer sampling, plant A and B were sampled again, in addition to the other two larger plants were also sampled (C and D). Still, none of these plants were positive for *Salmonella* and STEC by direct plating when sampled in spring and summer. In the summer, plant C produced samples that were positive for *Salmonella* when tested by direct plating; with a mean *Salmonella* count of 3.0±0.2 log CFU/m^3. The lack of *Salmonella* and STEC detection by direct plating in samples collected from beef harvesting establishments may be due to a low concentration of pathogens in the air of the beef plants in fall and winter, or even the presence of these pathogens in the viable but not culturable state (Rintala et al., 2008). Thus, direct plating should always combine with other methods for detecting airborne organisms qualitatively such as immunoassay or molecular-based methods.

Direct plating was not expected to be a detection method that could be compared to immunoassays or DNA-based methods. Instead, it was used to enable enumeration if the concentration of pathogens in the air was high enough to be detected. The detection limit of the direct plating method was 1.4 log/m^3. Therefore, the lack of detection on the plates does not indicate that *Salmonella* or STEC were not present in the air of the plants, but the pathogens, if

present, might have been below the 1.4- log CFU/m^3 detection limit. Meanwhile, direct plating may be an easy method for determining whether the concentration of pathogens is low or high and for quantitative purposes when the bacteria are at high levels. During sampling in summer, plant C tested positive by direct plating, which may be an indicator that the concentration of *Salmonella* was high enough to be detected by direct plating. In addition, this may indicate that the air of this plant has been contaminated with pathogens through aerosol during the slaughtering process, which supports one of our hypotheses. Since plant C was one of the large plants and the sampling was during summer time, this situation may give the indication of the possibility of the prevalence of *Salmonella* in the air of large meat establishments is more than small plants. The reason for differences in the prevalence of *Salmonella* and other pathogens in the air of meat processing areas may be due to the size of the plant and the processing activities. Usually, large plants have more slaughtering processes (number of animals to be killed and equipment used) and more worker activities that may contribute to greater air movement and generation of more bioaerosols. These assumptions are supported by Rahkio and Korkeala (1997) and Heldman (1974) who found a strong relationship between worker activities and airborne contamination. When all establishments were compared based on their size, it was found that there was a significant correlation between the size of the plant and the prevalence of *Salmonella* and STEC. In addition, the season also appeared to affect the prevalence of both *Salmonella* and STEC especially *Salmonella* in meat plant facilities. The larger number of positive samples for *Salmonella* occurred in summer as shown in Table 7. This indicated that the air of meat establishments might become contaminated with *Salmonella* and STEC more frequently during warmer seasons than during cooler seasons. Establishments likely need to take this information into consideration for strengthening sanitation programs during warm seasons.

Table 3. *Detection of STEC and* Salmonella *in aerosol samples collected at two small beef harvesting establishments and one feedlot in Texas during fall 2016 air sampling by direct plating*

Establishment	Number of samples	Direct plating count (log CFU/m^3)	
		STEC	*Salmonella*
A	12	<1.4/m^3[a]	<1.4/m^3
B	2	<1.4/m^3	<1.4/m^3
Feedlot[b]	2	<1.4/m^3	<1.4/m^3

[a]Not detected by a plate count method with a detection limit of 1.4 log CFU/m^3
[b]Feedlot that supplied beef cattle to establishment A.

4.3. Detection of STEC and *Salmonella* by CDx method

As mentioned before, CDx was used as one of the qualitative methods for analyzing the collected air samples. During fall sampling of establishment A and B, none of the samples collected during the two sampling days at establishment A tested positive for STEC or *Salmonella* by the CDx method. Similarly, samples collected from establishment B, and from the air in the feedlot that supplies cattle to establishment A, tested negative for STEC or for *Salmonella* by the CDx method (Table 4). Positive samples were not detected after enrichment and immunomagnetic separation as conducted in CDx method. However, both establishments A and B were positive for *Salmonella* and STEC for spring and summer of 2017 sampling when tested using the CDx method. In addition, plant C and D were sampled during spring and summer of 2017 and both plants tested positive for both *Salmonella* and STEC when tested using the CDx method. These establishments are considered large plants compared to plant A and B, and were sampled two times each. Establishment A and B tested negative for both pathogens in the fall season by the CDx method. After these negative results, the possibility that some pathogens present in the air sample might be injured or somehow not recoverable was

considered. The procedure recommended by the CDx manufacturer included an enrichment time of 18 hours for both pathogens. However, the approved methods were for food samples, and air samples could be different due to the lack of nutrients in the sample, even though PBS was used as sampling fluid. Therefore, the time of enrichment was extended to 36 hours and compared to 18 hours for all samples that were collected during spring and summer sampling for all plants. The data in Table 5 include the distribution of positive and negative air samples for *Salmonella* and STEC when enriching for 18 vs. 36 h. After changing the enrichment procedure from 18 to 36 h, the number of samples tested positive for both *Salmonella* and STEC in spring and summer seasons were increased significantly in all plants.

Table 4. *Detection of STEC and* Salmonella using Crystal Diagnostic (CDx) *in aerosol samples collected at two small beef harvesting establishments and one feedlot in Texas during fall 2016*

Establishment	Number of samples	*Crystal Diagnostic (CDx)* (log CFU/m^3)	
		STEC	*Salmonella*
A	12	<1.2/m^{3a}	<1.2/m^3
B	2	<1.2/m^3	<1.2/m^3
Feedlot[b]	2	<1.2/m^3	<1.2/m^3

[a]Not detected by the CDx method with a detection limit of 1.2 log CFU/m^3
[b]Feedlot that supplied beef cattle to establishment A.

Table 5. *Detection of STEC and* Salmonella *in air samples collected from beef harvesting establishments in Texas during spring and summer 2017 air sampling by Crystal Diagnostic when enriched for 18 and 36 hours.*

Plants	Organisms	Seasons	Detection by CDx method[a]					
			Dehiding		Fabrication		Chiller	
			18 h	36 h	18 h	36 h	18 h	36 h
A	*Salmonella*	Spring	+	+[b]	-[c]	-	-	-
	Salmonella	Summer	-	+	-	-	-	+
	STEC	Spring	-	+	-	+	-	-
	STEC	Summer	-	+	-	+	-	+
B	*Salmonella*	Spring	-	+	-	-	-	+
	Salmonella	Summer	-	+	-	+	-	-
	STEC	Spring	-	-	-	-	-	-
	STEC	Summer	-	+	-	+	-	+
C	*Salmonella*	Spring	-	-	-	+	-	+
	Salmonella	Summer	+	+	-	-	-	+
	STEC	Spring	-	+	-	+	-	+
	STEC	Summer	+	+	-	-	-	-
D	*Salmonella*	Summer	+	+	-	-	+	+
	Salmonella	Summer	+	+	-	-	+	+
	STEC	Summer	+	-	-	-	-	+
	STEC	Summer	+	+	+	+	+	+

[a] Detection of *Salmonella* and STEC in the air using CDx method
[b] The plus symbol (+) represents the sample tested positive with CDx analysis
[c] The minus (-) represents the sample tested negative with CDx analysis

The percentage of samples testing positive for *Salmonella* after 18 h of enrichment were 21.4, and for STEC were 17.9. However, after 36 h of enrichment, the percentages of positive samples were 57.1 and 60.7 for *Salmonella* and STEC respectively. Since 18 h enrichment samples also tested positive for spring and summer sampling, this may be an indicator that the concentration of bioaerosols was high enough for detection at shorter enrichment time during spring and summer seasons. The percentage of samples tested positive at 18 and 36 h of enrichment is provided in Table 4, and the percentage of positive samples at 18 h versus 36 h enrichment for both *Salmonella* and STEC is also shown in Figure 3. This is in disagreement with the CDx manufacturer's recommendation for testing food samples, and with the results of testing of ground beef during other activities in our laboratory, which produce positive detections with enrichment times of 18 h. The study was conducted to verify whether air samples indeed required more incubation time during enrichment included growth curves of *Salmonella* from air samples and testing after different times of enrichment. Based on the results from growth curves for *Salmonella*, the lag phase for the positive control was found to be around 2 h, whereas the lag phase for the naturally present *Salmonella* was around 6 h. Generation times were 25.8 min for the control and 126.5 min for the naturally occurring salmonellae. These results were significantly different ($P < 0.05$), and indicated that the airborne microorganisms may need a longer time to start doubling. The growth curves for both control and naturally occurring *Salmonella* are shown in Figure 4.

In addition, CDx testing of air samples after 18, 21, and 24 h enrichment resulted positive for *Salmonella* Typhimurium at all times. However, when non-inoculated *Salmonella* samples were tested, the results were all negative for 18 h enrichment, whereas 50% of the samples tested at 21 and 100 % of the samples tested at 24 h enrichment produced positive

detection results (the data are not shown in tabular form). The results obtained for *S.* Typhimurium and naturally present *Salmonella* from CDx testing were significantly different ($P<0.05$). This study confirmed that *Salmonella* present in beef hides needed more time for detection when testing within the detection system used in this study. However, further research would be needed to understand the reasons for this phenomenon. If the cells coming from the air, which likely can be traced back to the feedlot, were injured or had undergone viable but non-culturable stage as a result of the exposure to harsh environmental conditions, they might need more time to repair and become detectable. Even though the air sampler is designed to protect the bacteria in the sample from osmotic shock, if they were already injured when present in the air, they will likely continue to hold the injury in the sample that is protected in the air sampler. This hypothesis will be tested in future research since an understanding of bioaerosols in beef harvest establishments is an area of research that needs to be further explored.

Based on these results, the detection of airborne *Salmonella* by CDx may need between 22-24 h enrichment times.

Table 6. *The Samples tested positive for* Salmonella and *STEC at 18 & 36 hours enrichments using Crystal Diagnostic CDx method for spring and summer season sampling*

The plant	Organisms	Percentage of positive samples at 18 h enrichment[a]		Percentage of positive samples at 36 h enrichment[b]	
		Spring	Summer	Spring	Summer
A	*Salmonella*	20	0.0	40.0	40.0
	STEC	0.0	0.0	40.0	60.0
B	*Salmonella*	0.0	0.0	40.0	66.6
	STEC	0.0	0.0	20.0	100.0
C	*Salmonella*	0.0	33.3	66.6	66.6
	STEC	0.0	0.0	100.0	33.3
[d]D	*Salmonella*	NS	66.6	NS	66.6
	STEC	NS	66.6	NS	66.6

[a]The number of positive *Salmonella* or STEC samples at 18 h enrichment for each plant
[b] The number of positive *Salmonella* or STEC samples at 36 h enrichment for each plant
[c] the percentage of *Salmonella* or STEC samples at 18 h or 36 h enrichment.
[d] plant D was only sampled during summer two times, but not in the spring. Therefore, no percentage was reported for this plant in spring. "NS" refers to not sampled.

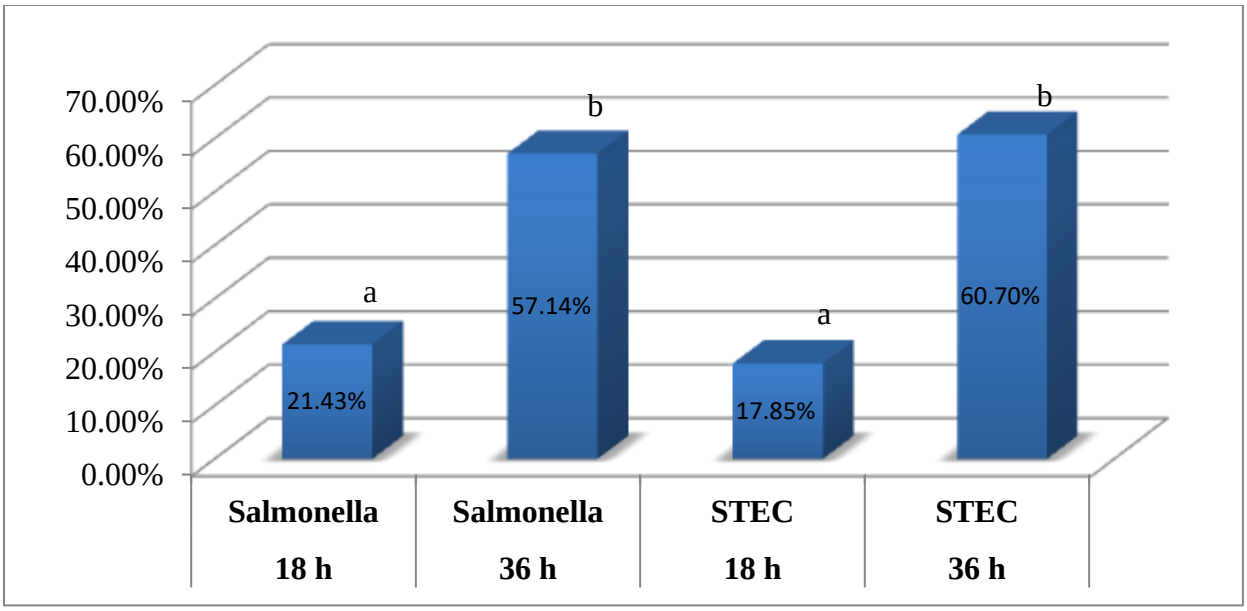

Figure 3. *The percentage of* Salmonella *and STEC tested positive by CDx immunoassay method at 18 h enrichment and 36 h, The percentage of both* Salmonella *and STEC was significantly different (P<0.05) when the air samples enriched for 36 h than enrichment for 18 h. Column with same letters (a, or b) are not different.*

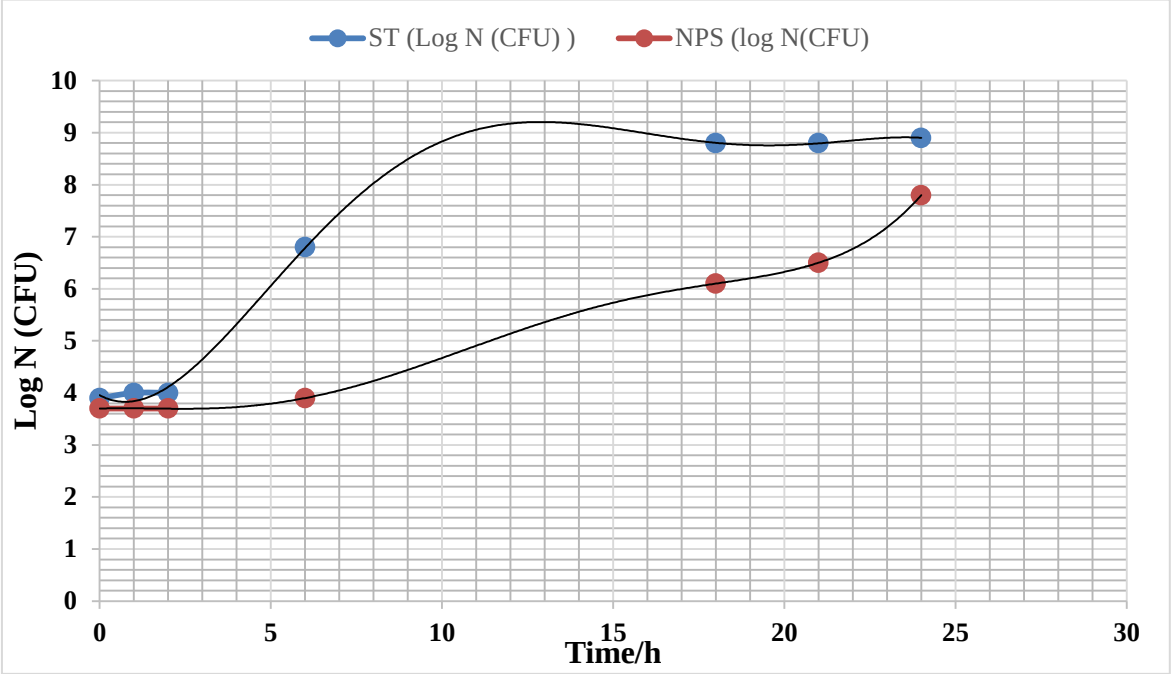

Figure 4. *Growth curve of* Salmonella *Typhimurium (ST)(control) and naturally present* Salmonella *(NPS) in the beef hide at a constant temperature (37 °C). These two growth models were used after aerosolization of the naturally present Salmonella to compare them. The generation time for naturally present* Salmonella *was 126.5 min and the lag phase around 6 h, compared to* Salmonella *Typhimurium (control), for which the generation time was 25.8 min and the lag phase 2h, significantly different ($p<0.05$).*

Table 7. *The effect of season on the prevalence of* Salmonella *and STEC in the air of meat establishments*

	Detection by CDx method	
The season	Total No. of Samples[a]	Percentage of Positives[b]
Fall	20	0A[c]
Spring	26	46B
Summer	30	70C

[a]The total number of air samples for all plants in fall, spring, and summer seasons
[b]The percentage of positive samples for *Salmonella* and STEC for all plants
[c]In the same columns, number with the different letter A, B or C are different ($P < 0.05$).

In addition, it was found that the presence of *Salmonella* and STEC also affected by the location of sampling within the same establishment. Positive samples were found in all areas that were sampled during the different time within different plants, which may be indicated that bioaerosols are present at different processing areas at different levels depending on the other conditions mentioned before such as worker activities, the air movement, the size of the plant, and the time of sampling. The presence of airborne bacteria and generating of bioaerosols at various processing areas was also reported by Jericho et al., (2000). When compared different areas of air sampling, positive detection of *Salmonella* and STEC was mostly found in dehiding area as shown in Table 8. This is in agreement with the results obtained by Schmidt et al., (2012) and Rahkio and Korkeala (1997) who showed that the higher positive samples for *E. coli* and *Salmonella* were in dehiding area than other areas such as washing. The presence of *Salmonella* and STEC in dehiding area is expected due to the fact that all slaughtering process, worker activities and washing are performed in this area, which may lead to the generation of more bioaerosols from worker activity and the significant aerosolization from high speed carcass splitting and wastewater more than the other areas. Prendergast et al. (2004) also reported a similar observation and demonstrated that contamination of the airborne organisms was higher at carcass splitting area than washing area. The numbers of positive samples at chiller area were also high compared to the number of positive samples in fabrication and stunning areas. The presence of *Salmonella* and STEC in chiller area may be due to the potential for aerosols being carried from the kill floor to chiller room during carcass transferring to chiller by the air movement. In addition, worker activities and personal movement are contributing to transfer of these bioaerosols from dirty areas such as dehiding to clean areas such chiller room. This observation is in agreement with Rahkio and Korkeala (1997) who concluded that the potential

contamination of clean area from the unclean area by airborne organisms associated with worker activities and personal movement.

Although the presence of *Salmonella* and STEC was confirmed in the air of the dehiding area and chiller, as well as in other areas such as fabrication room and stunning area, none of these positive samples was positive for *E. coli* O157: H7, as observed during confirmation testing of positive samples, when STEC isolates were tested by biochemical tests and then subjected to serological confirmation for *E. coli* O157: H7. However, the isolates obtained from STEC positive samples were confirmed as STEC when testing for the presence of *stx* and *eae* genes by conventional PCR.

Table 8. *Incidence of* Salmonella *and STEC in air collected from different areas of the beef carcass processing*

	Detection by CDx. method	
The plant	Total No.[a] of samples	The percentage of Positives[b]
Dehiding	20	65A[c]
Fabrication	20	35B
Chiller	20	55A
Stunning	8	0C
Evisceration	8	25B

[a]The total number of air samples for all plants in fall, spring, and summer seasons
[b]The percentage of positive samples for *Salmonella* and STEC for all plants
[c]In the same columns, number with the same letter A , B or C are not different ($p>0.05$).

The results of this study indicated the association between carcasses contamination and airborne bacteria through generating bioaerosols. This was in agreement with Pearce et al., (2006) who exhibited a strong association between the carcass and microbiological contamination of the air of pork slaughter plant. The level and concentration of bioaerosols may be dependent on the area of sampling within the same plant, the size of meat plant, and the time of sampling in the year. More research is needed to confirm these finding with more plants, and extending the time of sampling to determine the actual relation between the season and prevalence of the airborne organisms especially pathogenic *Salmonella* and *E. coli*.

4.4. Detection of coliform bacteria in the air of meat plant establishments

In addition to our interest to detect *Salmonella* and STEC in the air of meat plant facilities, we also were interested to see the level of coliform bacteria in the air of meat plants that were sampled for pathogens. Coliforms are expected to be present in the air and can be counted, and in case the pathogens could not be detected, the coliform bacteria counts may be used as indicators for the presence of bioaerosols in meat plants. Out of four plants that were sampled during three consecutive seasons, total coliform bacteria were detected at plant B at different areas. The mean log CFU/m^3 of total coliform bacteria detected at dehiding, stunning and evisceration was 2.9, 2.9, and 1.8 log CFU/m^3 respectively. The mean of the log CFU/ m^3 of *E. coli* detected at dehiding area was 1.8 log CFU/m^3 in the fall season. The coliform bacteria also were detected at plant C and D during summer sampling and were also at dehiding area. The mean log CFU/ m^3 of total coliforms and *E. coli* for both plant C and D at dehiding area were (4.3, 3.2) and (3.4, 2.5) log CFU/ m^3 respectively. Meanwhile, pathogens were detected in different areas. The detection of pathogens at different areas during spring and summer air

sampling may be due to the extension of enrichment time from 18 to 36 h, where the concentration of airborne organisms are low and probably not detected by cultural methods before enrichment, and even 18 h enrichment was not sufficient for low concentrations. And this might be the reason for coliforms were not detected at other areas of air sampling. In addition, the presence of coliforms at these plants may not always indicate the presence of pathogens; sometimes their prevalence just gives the indication of unsanitary condition, which is not necessarily related to pathogens. Or, their presence may indicate the presence of pathogens; the results are shown in Table 9.

Table 9. *Detection of coliform bacteria in air samples collected from beef harvesting establishments in Texas during three seasons*

The plant	Seasons	Coliforms	(Log CFU/m^3) at sampling different areas [a]				
			Stunning	Dehiding	Evisceration	Chiller	Fabrication
A	Fall	Total	<0.5[b]	<0.5	<0.5	<0.5	<0.5
		E. coli	<0.5	<0.5	<0.5	<0.5	<0.5
	Spring	Total	<0.5	<0.5	<0.5	<0.5	<0.5
		E. coli	<0.5	<0.5	<0.5	<0.5	<0.5
	Summer	Total	<0.5	<0.5	<0.5	<0.5	<0.5
		E. coli	<0.5	<0.5	<0.5	<0.5	<0.5
B	Fall	Total	2.9	2.9	1.8	<0.5	<0.5
		E. coli	<0.5	1.8	<0.5	<0.5	<0.5
	Spring	Total	<0.5	<0.5	<0.5	<0.5	<0.5
		E. coli	<0.5	<0.5	<0.5	<0.5	<0.5
	Summer	Total	<0.5	<0.5	<0.5	<0.5	<0.5
		E. coli	<0.5	<0.5	<0.5	<0.5	<0.5
C	Spring	Total	<0.5	<0.5	<0.5	<0.5	<0.5
		E. coli	<0.5	<0.5	<0.5	<0.5	<0.5
	Summer	Total	<0.5	4.3	<0.5	<0.5	<0.5
		E. coli	<0.5	3.2	<0.5	<0.5	<0.5
D	Summer	Total	<0.5	3.4	<0.5	<0.5	<0.5
		E. coli	<0.5	<0.5	<0.5	<0.5	<0.5
D	Summer	Total	<0.5	2.5	<0.5	<0.5	<0.5
		E. coli	<0.5	<0.5	<0.5	<0.5	<0.5

[a]The Log CFU/m^3 of coliforms or *E. coli* samples at each area of sampling for each season
[b]The 0.5 is a detection limit for coliform bacteria, therefore < 0.5 refers to none detectable coliforms at 0.5 CFU/m^3 . The absolute values without the less than (<) are the log CFU/ m^3 detected in the air of meat establishments.

4.5. Detection of STEC and *Salmonella* by RT-PCR analysis (Aerosol Technology Laboratory results)

For RT-PCR detection, the most common genes associated with virulence factors for STEC and *Salmonella* were used as targets. Therefore, for STEC, *stx* and *eae* primers were used, whereas the *inv*A gene was used for *Salmonella*. Although different genes can be used to detect *Salmonella*, *inv*A gene is used for this study because it is a common virulence factor for most *Salmonella*. In addition, this gene contains sequences unique to this gene and found it an appropriate gene for PCR analysis (Shanmugasamy et al., 2011). In addition, each plant was sampled differently, for example, plant A was sampled four times, and sometimes for 2-3 days, which was the completely different situation for other plants. This variation in sampling times and dates was because it was difficult to obtain permission from the meat plants at the same time. In addition, during fall sampling, plant A was sampled twice and for 2-3 days each time because we wanted to design the best sampling protocol and ensure that the required volume of air was collected. Therefore, the following discussion of results for RT-PCR is discussed in separate subsections.

4.5.1. Establishment A RT-PCR data analysis for STEC (*stx* and *eae*) genes

For plant A**,** The bioaerosols collected during a 3-day period at establishment A in the fall, and one day for spring and summer were analyzed. The number of samples tested positive for STEC genes were recorded at each location. The number of positive samples tested positive for *stx* and *eae* genes were obtained using RT-PCR. There were significant differences ($P<0.05$) between the number of samples tested positive for *stx* genes and the number of samples tested positive for *eae*. The number of samples tested positive for *eae* gene were much lower compared

to the number of samples positive for *stx* gene. The samples that were positive for STEC *eae* gene in plant A were found mostly in dehiding area, and only two samples found to be positive for *eae* in the chiller. Most of the STEC isolates were *eae* negative; only a few isolates were detected, they were associated with plant A and C, and they were *stx* negative. The presence of these *stx* negative, *eae* positive isolates could be obtained from STEC strains that have lost their phage-encoded *stx* genes (Mellmann et al., 2005). The detection of more airborne organisms with positive *stx* genes indicated that these microorganisms are lacking the ability of attachments. In other words, the presence of these airborne organisms may not pose great risks since they are able to produce a toxin, but they are lacking the ability to attach to the host cell.

4.5.2. Establishment B RT-PCR data analysis for STEC (*stx* and *eae*) genes

For plant B, the bioaerosols were collected during a one-day period for each season (fall, spring, and summer). A number of positive samples were recorded. The same observations as plant A were observed regarding the differences between samples tested positive at each location of sampling, as most positive samples were found in dehiding area. There were significant differences ($P<0.05$) between samples tested positive for STEC *stx* and *eae* genes and between the number of the positive sample tested for *eae* genes only. In fact, none of the samples were positive for *eae* in this plant.

4.5.3. Establishment C RT-PCR data analysis for STEC (*stx* and *eae*) genes

Plant C was sampled during spring and summer, and the results obtained from this plant differed from the other two plants, in which there were no significant differences ($P>0.05$) between the number of samples tested positive for *stx* and *eae* genes. On the other hand, there

were still differences between the number of the sample tested positive at each location. Most of the positive samples for both *stx* and *eae* were found in dehiding and chiller areas. Plant C was one of the large plants that were sampled for two days each time during spring and summer, which indicated that longer sampling time and larger plants may be the reason for finding more *eae* positive isolates.

4.5.4. Establishment D RT-PCR data analysis for STEC (*stx* and *eae*) genes

Plant D was sampled two times during summer, none of the samples were tested positive for *eae* gene in this plant. While positive samples for *stx* were detected at different locations including dehiding, chiller, and fabrication room. Interestingly, a number of samples tested positive of *stx* at dehiding area were the same at chiller area. This observation is important because it gives the indication that chiller area becomes highly contaminated since the number of isolates that were detected was similar to the one of the dirty areas such as dehiding. This may be possible because of the size of the plant since the plant C was also one of the large plants; in addition, it may be affected by the season since the sampling was during summer. The data and information for all plants that were analyzed by RT-PCR for *stx* and *eae* are shown in Table 10. For more clarification, the number STEC positive for *stx* or *eae* at each location also graphed, these are shown in Figure 5 and Figure 6.

4.5.5. All establishment RT-PCR data analysis for *Salmonella*

The *invA* gene was used to detect *Salmonella* by RT-PCR. Again different levels of positive samples were obtained at each location for each plant during different seasons. The number of samples tested positive for *Salmonella* were significantly higher at plant A compared

to other plants. Positive samples for *Salmonella* were obtained for plant A at all locations including dehiding, fabrication, chiller, eviscerating, and stunning. Plant B only tested positive for *Salmonella* at dehiding, chiller, and stunning area, but was negative at fabrication area. Plant C was tested positive for one sample at each location including dehiding, fabrication, and chiller. Finally, plant D was positive for *Salmonella* only at dehiding area. The reason for this variation might be due to the number of times each plant was sampled, where plant A was sampled four times, plant B three times, and each of plant C and D was sampled just two times, the data in Figure 7 are shown the number of *Salmonella* tested positive by RT-PCR.

Table 10. *Detecting of STEC positive for stx and eae genes during fall, spring and summer sampling by RT-PCR*

Plants	Locations	No. of positive samples during all seasons	
		stx[a]	*eae*[a]
A	Dehiding	9	2
	Fabrication	6	2
	Chiller	9	2
	Eviscerating	6	2
	Stunning	7	2
B	Dehiding	3	0
	Fabrication	2	0
	Chiller	2	0
	Eviscerating	3	0
	Stunning	0	0
C	Dehiding	5	6
	Fabrication	4	4
	Chiller	5	4
	Eviscerating	0	0
	Stunning	0	0
D	Dehiding	3	0
	Fabrication	2	0
	Chiller	3	0
	Eviscerating	0	0
	Stunning	0	0
	Total	69	24

[a] represent the number of positive samples for *stx* and *eae* during fall of 2016, and spring and summer of 2017. (Aerosol Technology Laboratory results)

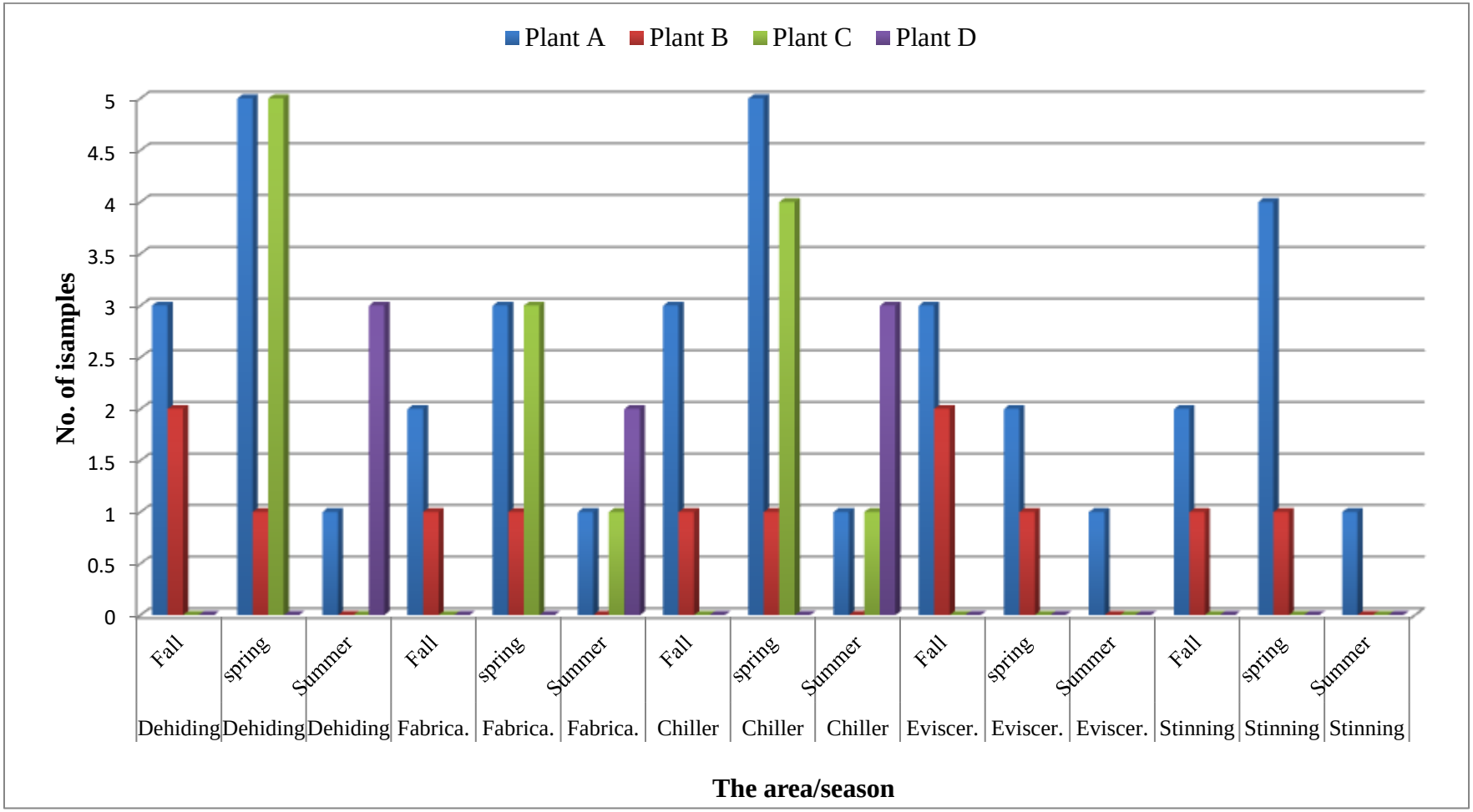

Figure 5. *Detection of STEC with stx gene by RT-PCR during fall of 2016 and spring and summer of 2017. The higher number of positive samples for STEC (stx gene) were detected at plant A (blue), then plant C (green) was the second plant that tested the higher number of STEC with stx gen, especially during spring sampling, plant D (purple) was the third plant in testing STEC positive samples for stx. The lower positive STEC samples for stx were obtained by plant B (red). Plant A was significantly different ($P<0.05$) than all other plants (B, C, and D). (Aerosol Technology Laboratory results)*

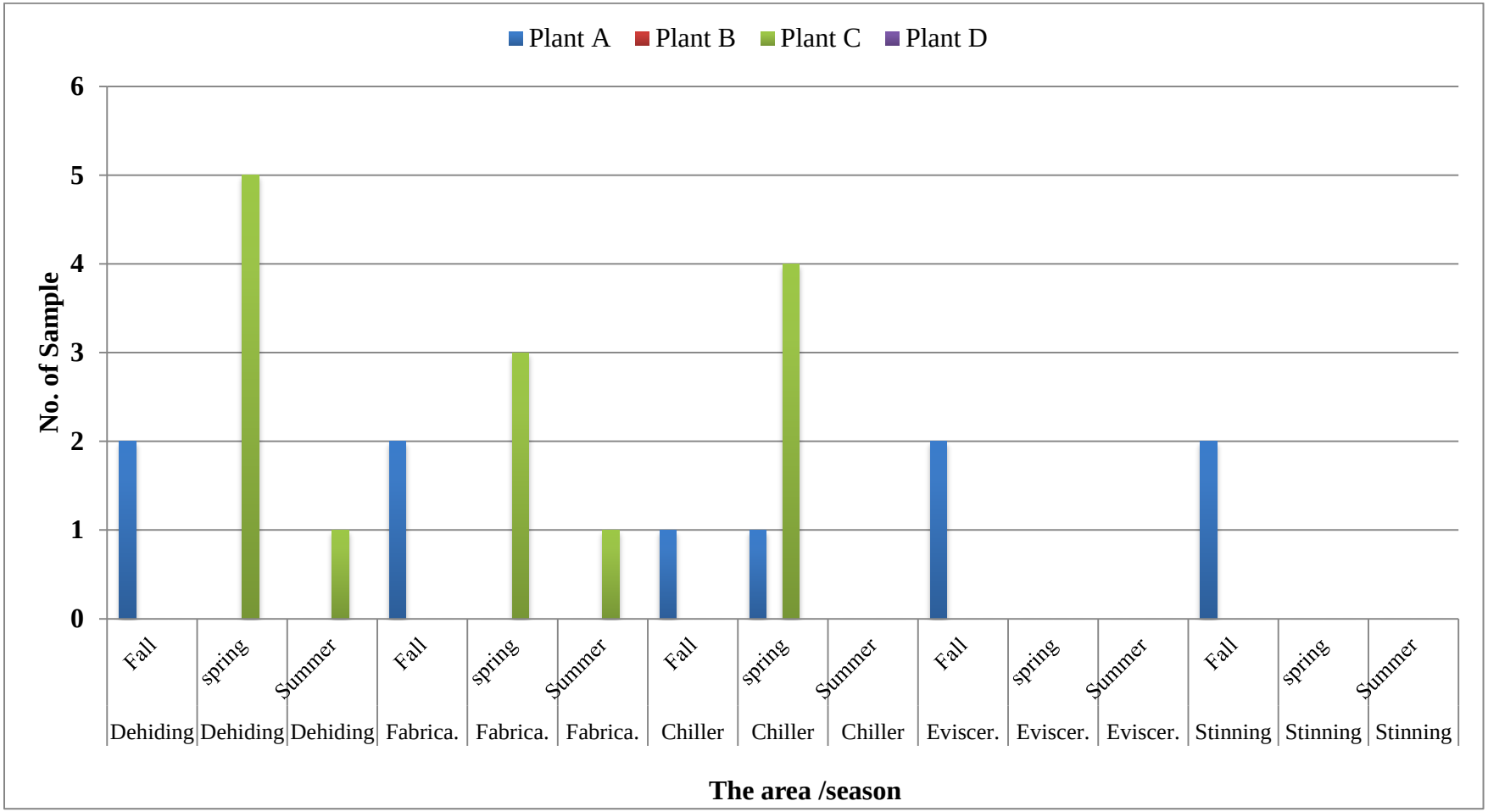

Figure 6. *Detection of STEC with eae gene by RT-PCR during fall of 2016 and spring and summer of 2017. The higher number of positive samples for STEC (eae gene) were detected at plant C (green) during spring season, then plant A (blue) was the second plant that tested for STEC with eae gen, especially during fall sampling, plant D (purple) and plant B (red) were both tested negative for STEC eae gene. However, Plant C was not significantly different (P>0.05) from Plant A. (Aerosol Technology results)*

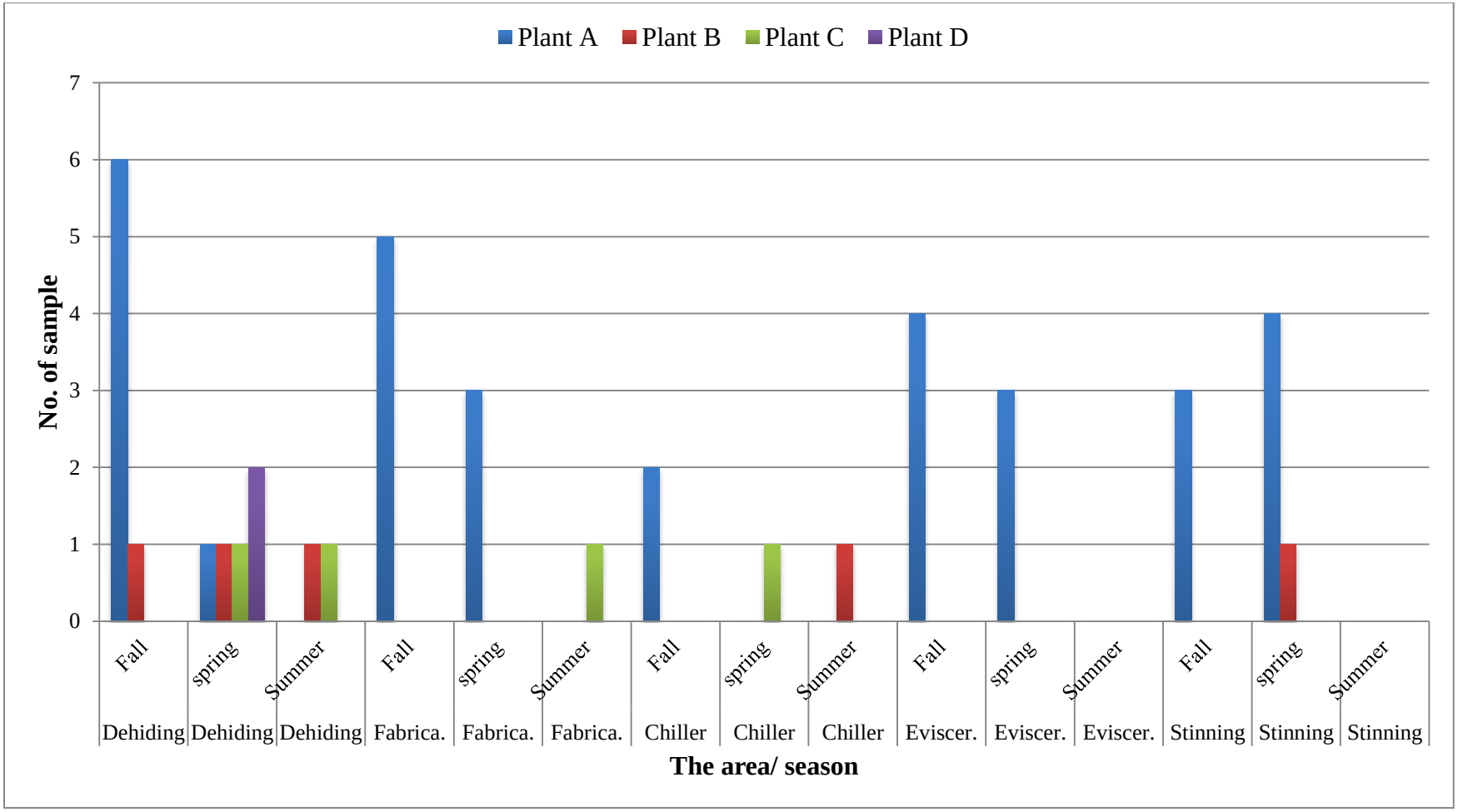

Figure 7. *Detection of Salmonella with invA gene by RT-PCR during fall of 2016 and spring and summer of 2017. The higher number of positive samples for Salmonella (invA gene) were detected at plant A (blue), the plant B (red), plant C (green), and plant D (purple) were all testes positive at similar levels of positive samples for Salmonella during all seasons and at were different at each location of sampling. Plant A was significantly different (P<0.05) than all other plants (B, C, and D). (Aerosol technology Laboratory results)*

4.6. Comparison between RT-PCR and CDx methods

RT-PCR Molecular-based method to detect *Salmonella* and STEC in the air of meat abattoirs in Texas was compared with CDx immunoassay-based methods. The results from both methods for two small and two large plants in spring, and summer season were used to compare between these methods. The results for *Salmonella* and STEC were tested positive with RT-PCR and CDx for both spring and summer seasons. However, the proportion of samples tested positive for each method was different among the plants. The percentages for each pathogen or each STEC genes were different. When tested with RT-PCR, The percentage of samples tested positive for *Salmonella* and STEC was (37.5) and (65.0) respectively, while with CDx method, the percentage of samples tested positive at 18 h enrichment was (21.4) and (17.9) for *Salmonella* and STEC respectively, and (57.2) and (60.7) at 36 h enrichment. The RT-PCR assay detected higher proportion ($P< 0.05$) of positive samples tested for *Salmonella* and STEC than the CDx method when samples enriched for 18 h. However, when the enrichment time was extended, the CDx assay detected higher proportion ($P<0.05$) of positive samples tested for *Salmonella*. Meanwhile, both methods detected about the same proportion of positive samples for STEC ($P>0.05$). The reason behind this variation between two methods may be due to the factors that affect each method individually. For CDx method, the results might be affected by antigen sharing with other microorganisms present in the testing sample. Thus sometimes, the results ended up with the false negative. In addition, when air samples enriched for 18 h, the number of positive samples was either absent or very low, which may be indicated that 18 h enrichment was not sufficient for air samples to recover *Salmonella* or STEC to be detected by CDx. In case of RT-PCR, positive results might be indicated of none viable organisms because not all positive *Salmonella* or STEC detected by RT-PCR are viable as RT-PCR detects both

viable and none viable organisms, the presence of inhibitors might be affected the number of positive samples. All *Salmonella* positive samples were confirmed with XLT4 media and biochemical tests. STEC positive samples were confirmed with Possé agar. In conclusion, in some samples that were negative by one test, was positive by the other method, thus indicating that both methods are necessary to provide an accurate detection of the presence of *Salmonella* and STEC for sampling the air of meat facility plants.

4.7. *Salmonella* and STEC confirmation and characterization

The results for samples positive for *Salmonella* and STEC by RT-PCR or CDx methods are shown in subsections related to this section. These include the results from conventional PCR that used for STEC confirmation and BAX PCR System that was also used for further confirmation of both *Salmonella* and STEC. Finally, the PFGE was done for typing of positive isolates to see if a group of these isolates is related to each other at different areas of sampling.

4.7.1. Confirmation with conventional PCR

Presumptive STEC isolates were confirmed using gel-based PCR to detect *stx* and *eae* genes. The PCR products confirmed the presence of STEC, the majority of samples were positive for *stx*, some of the samples were positive for both *stx* and *eae* genes, and few samples were positive for *eae* only. The amplicon size was ranging between 102-132 bp. The results indicated the presence of STEC as bioaerosols in meat slaughterhouses, which support our first objective. The pattern of PCR products for STEC samples from spring and summer is shown in Figure 8.

4.7.2. Confirmation with BAX PCR System

The confirmation of all *Salmonella* and STEC isolates was conducted using a BAX System at FSITRL. A total of 54 *Salmonella* isolates were screened with this method and the results were positive for 50 isolates of *Salmonella*. Only 4 isolates were negative for *Salmonella*, and they were positive for STEC. While for STEC isolates, out of 48 isolates, 8 isolates were negative for STEC with this system. And therefore were discarded. Most of the isolates were positive for *stx* gene whereas only three isolates were positive for both *stx* and *eae*. Similar results were obtained by RT-PCR, where the majority of STEC positive samples were *stx* positive, only a few samples tested positive for *eae* by RT-PCR. This screening test was done to ensure the identity of isolates whether they are *Salmonella* or STEC prior to the fingerprinting using PFGE for setting the PFGE conditions accordingly.

4.7.3. Illumina sequencing

All *Salmonella* and STEC samples collected at meat plants and tested positive by RT-PCR analysis were confirmed by Illumina next generation sequencing. The results were handled by the Aerosol Technology Laboratory and were analyzed. The result obtained from sequencing confirmed the presence of *Salmonella* and STEC at the air of meat abattoirs, and the potential contamination due to bioaerosols.

4.7.4. Pulse Field Gel Electrophoresis (PFGE)

Pulse Field Gel Electrophoresis is used for typing the positive isolates from *Salmonella*, STEC, and coliforms. For the current research, the purpose of bacterial typing is to determine if a group of bacteria are related and represent the same strain. The information about bacterial

typing is useful for understanding and controlling the spread of bacteria, and to determine whether the results support our hypothesis that airborne microorganism may transfer from unclean areas to clean areas such as a chiller. After the results were obtained from the Food Safety and Intervention Technology Research Lab of the USDA-Agriculture Research Service (FSITL), the data were analyzed and results are showed that within each plant at different locations all *Salmonella* isolates were the same pulsotype, except for one isolate that was came from plant B and specifically from dehiding area shared the same pulsotype with plant A. This phenomenon might be due to mixing that the isolate unintentionally labeled as came from plant B or the isolate came from animals that originate from the same feedlot and acquired the same organisms, the results are presented in Table 11. For STEC isolates, similar results were obtained for all plants. A total of four different pulsotype for different plants (A-D) that isolates originally came from. The results indicated that within each plant and different locations, STEC found to be the same type. This again further supports our hypothesis that the presence of airborne at meat processing plant has a potential risk of contamination of carcass by transferring bioaerosols from dirty areas into clean areas, the results are shown in Table 12. While the results of PFGE for coliforms were different, as the results showed two different pulsotype of coliforms in Plant B, the isolates that were from dehiding was different from pulsotype from fabrication area. While the isolates from each of plant C or D was the same coliforms' pulsotype. The results of PFGE pattern for *Salmonella*, STEC, and coliforms are shown in Figure (9-11). The fact that all isolates of *Salmonella* and STEC shared the same pulsotype, is further confirmed carcasses could be contaminated by bioaerosols containing bacteria that were generated during dehiding process. Thus, a potential contamination of clean areas by transferring bioaerosols from unclean areas is possible. This is in agreement with Schmidt et al., (2012), who demonstrated a strong association

between carcasses contamination and airborne bacteria during hide removal. Based on the results obtained from the PFGE analyses, it was observed that each meat establishment has only one pulsotype for each *Salmonella* and STEC isolates. This is indicated that all *Salmonella* or STEC are the same strain within the same plant. While this observation may be possible, no literature was found to support this finding. One possible explanation of the presence of one pulsotype of each pathogen within the same beef establishment could be traced back to the animals, perhaps being infected with one type (strain) of *Salmonella* or STEC. Another clarification is that for PFGE testing, three isolates were taken from each plate and may be the most prevalent DNA was selected.

***Figure 8**. PCR products for STEC samples from spring sampling for plant A and B (top image), and PCR products for STEC from summer sampling for plant C and D (bottom image). The amplicon size for spring sampling was 132 bp Stx primers and 102 bp for eae promers, similar amplicon size was obtained.*

Table 11. *Pulse Field Electrophoresis (PFGE) for* Salmonella *isolates and resulting pulsotypes*

Plants	Areas	No. isolates subjected to PFGE	No. *Salmonella* isolates corresponding to 4 pulsotypes (PT)			
			PT1[a]	PT2	PT3	PT4
Plant A	Dehiding	11	11[b]	0	0	0
	Evisceration	3	3	0	0	0
	Chiller	3	3	0	0	0
	Fabrication	0	0	0	0	0
Plant B	Dehiding	4	1[c]	3	0	0
	Evisceration	0	0	0	0	0
	Chiller	1	0	1	0	0
	Fabrication	3	0	3	0	0
Plant C	Dehiding	4	0	0	4	0
	Evisceration	0	0	0	0	0
	Chiller	6	0	0	6	0
	Fabrication	3	0	0	3	0
Plant D	Dehiding	3	0	0	0	3
	Evisceration	0	0	0	0	0
	Chiller	9	0	0	0	9
	Fabrication	0	0	0	0	0

[a] The abbreviation of pulsotype; four pulsotype for STEC isolates in four plants
[b] Refers to the number of isolates found in that specific area.
[c] One isolate from plant B at dehiding area share the same pulsotype with plant A. (The Food Safety and Intervention Technology Research Lab of the USDA-Agriculture Research Service (FSITL) results)

Table 12. *Pulse Field Electrophoresis (PFGE) for STEC isolates and resulting pulsotypes*

Plants	Areas	No. isolated subjected to PFGE	No. STEC isolates corresponding to 4 pulsotypes (PT)			
			PT1[a]	PT2	PT3	PT4
Plant A	Dehiding	2	2[b]	0	0	0
	Evisceration	0	0	0	0	0
	Chiller	2	2	0	0	0
	Fabrication	2	2	0	0	0
Plant B	Dehiding	2	0	2	0	0
	Evisceration	3	0	3	0	0
	Chiller	2	0	2	0	0
	Fabrication	3	0	3	0	0
Plant C	Dehiding	7	0	0	7	0
	Evisceration	0	0	0	0	0
	Chiller	3	0	0	3	0
	Fabrication	2	0	0	2	0
Plant D	Dehiding	1	0	0	0	1
	Evisceration	0	0	0	0	0
	Chiller	4	0	0	0	4
	Fabrication	5	0	0	0	5

[a] The abbreviation of pulsotype; four pulsotype for STEC isolates in four plants
[b] Refers to the number of isolates found in that specific area. (The USDA-Agriculture Research Service ((FSITL) results)

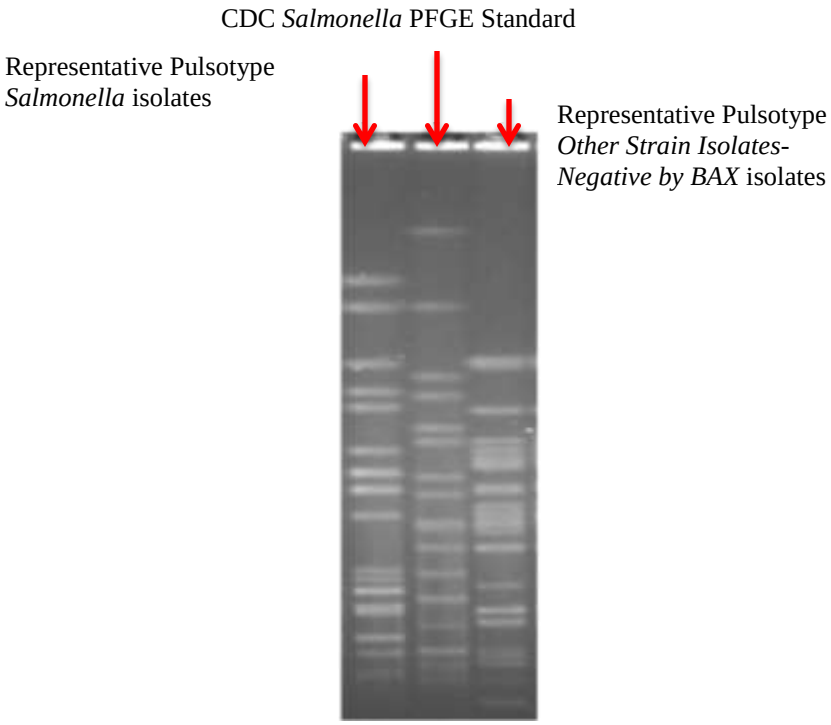

Figure 9. *PFGE patterns of* Salmonella *chromosomal DNA restriction fragments resolved in 1% Seakem Gold agarose in 0.5x TBE buffer for* E. coli *DNA digested with Xbal (pulse time, 2.2 to 54.2 s; running time, 19 h). The sizes of the fragments are indicated in kilobases. These are* Salmonella *isolates from air samples of meat establishments that are collected during fall of 2016, and spring and summer of 2017.* (FSITL) results)

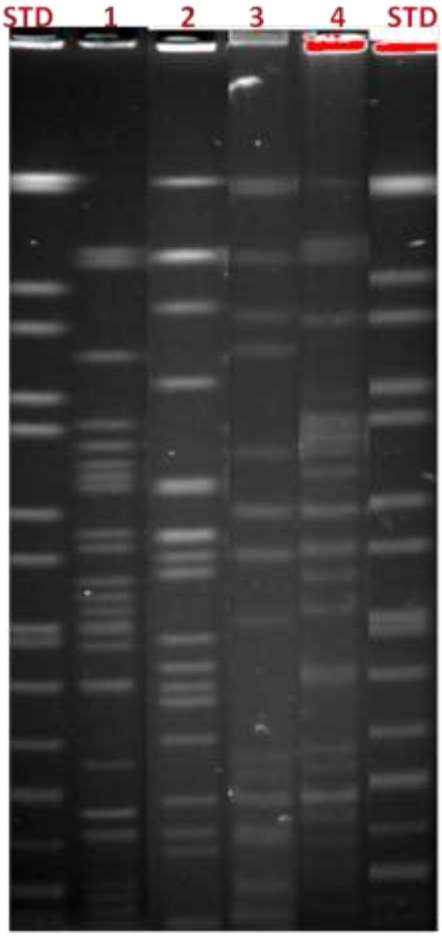

Figure 10. *PFGE patterns of STEC chromosomal DNA restriction fragments resolved in 1% Seakem Gold agarose in 0.5x TBE buffer for* E. coli *DNA digested with Xbal (pulse time, 2.2 to 68.8 s; running time, 19 h). The sizes of the fragments are indicated in kilobases. These are STEC isolates from air samples of meat establishments that are collected during fall of 2016, and spring and summer of 2017.* (FSITL) results)

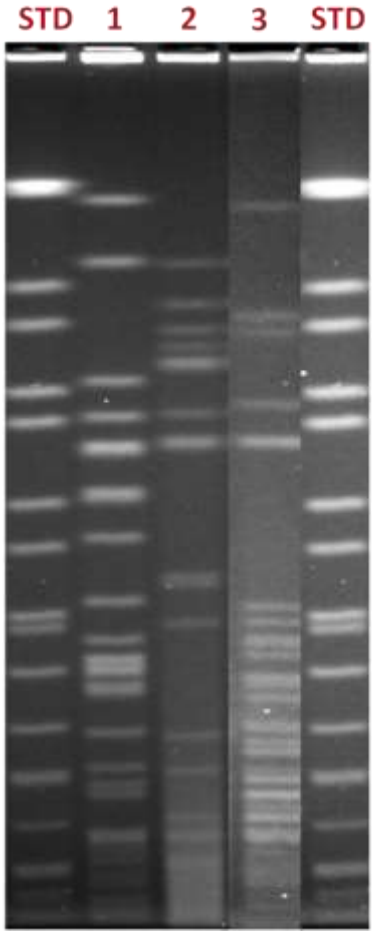

Figure 11. *PFGE patterns of coliforms chromosomal DNA restriction fragments resolved in 1% Seakem Gold agarose in 0.5x TBE buffer for E. coli DNA digested with Xbal (pulse time, 2.2 to 68.8 s; running time, 19 h). The sizes of the fragments are indicated in kilobases. These are coliform bacteria isolates from air samples of meat establishments that are collected during fall of 2016, and spring and summer of 2017.* (FSITL) results)

CHAPTER V

CONCLUSIONS

5.1. Conclusions

The results of this study provide information about the likelihood of air transfer of potential pathogens to fresh beef via bioaerosols formed during slaughter processing.

Except for plant C during the summer sampling, direct plating to detect *Salmonella* and STEC was negative for all plants and all seasons throughout this study. The lack of detection of *Salmonella* and STEC on most samples by direct plating may be due to the low concentration of pathogens in the air of the beef plants. Detection of *Salmonella* by direct plating at one-meat establishments during summer may indicate that the concentration of *Salmonella* was relatively high in bioaerosols. In addition, the presence of total coliform bacteria and *E. coli* during summer sampling may also indicate a larger concentration of microorganisms in the air of these plants. Total coliform bacteria were detected in plant B at different areas. The mean log CFU/m^3 of total coliform bacteria detected at plant B at dehiding, stunning and evisceration was 2.9, 2.9, and 1.8 log CFU/m^3 respectively. And the means log CFU/ m^3 of *E. coli* detected at dehiding area was 1.8 log CFU/m^3 in the fall season. While, the means log CFU/ m^3 of total coliforms and *E. coli* for both plant C and D during summer sampling (4.3, 3.2) and (3.4, 2.5) log CFU/ m^3 respectively. However, *E. coli* O157:H7 was not found in any air sample tested by direct plating. The analysis conducted by CDx produced negative results for both pathogens in plant A and B during fall sampling. This finding presented an opportunity to reevaluate the CDx method applied to air samples. It was found that when the time of enrichment was extended to 36 h as opposed to the recommended 18 h, the number of positive samples increased significantly. This

permitted to propose the possibility that enrichment time be extended when testing similar types of samples. Meanwhile, the RT-PCR method was successful in detecting both *Salmonella* and STEC in all plants during all seasons. After enrichment, the number of positive samples detected by CDx method was comparable to the number of positive samples detected by the RT-PCR method. On the other hand, most collected samples from different plants tested positive for STEC *stx* gene, while only a few samples in two meat plants were tested positive for *eae* gene when tested by RT-PCR. This provides an indication that the prevalence of the STEC with *stx* gene may be higher than the prevalence of STEC with *eae* gene in meat plants. The results indicated that the presence of *Salmonella* and STEC in the air of meat establishment was not only dependent on the location of the physical areas where carcasses are processed, but also was dependent on the size of plant, and season. A significant correlation was found between the presence of STEC and *Salmonella,* and the size of plant, season and the location of sampling. When positive isolates of *Salmonella* and STEC were fingerprinted by Pulse Field Gel Electrophoresis (PFGE**),** it was found that within each plant, the DNA of *Salmonella* or STEC is the same at different locations. The relation between positive isolates at different locations, support our assumption for this study, which is the potential transfer of aerosols containing pathogenic bacteria from unclean areas such as dehiding to clean areas such as a chiller. More research is needed to support our findings. It is also suggested that the sampling time for future research be extended to be years of sampling including all seasons to determine the effect of season.

5.2. Future research

More research in this area should be conducted in the all types of slaughtering establishments including; small, medium and large plants to evaluate the effect of the size of meat plants on the prevalence of airborne microorganisms. More research is needed to evaluate the incubation times (18 and 36 h) of enrichment media and to validate the required enrichment time, the enrichment may be less than 36 h, but should be more than 18 h. The air sampling should be considered to be conducted in different States in the US or even outside the US, and it should be taken throughout the year and for a longer time may be years to determine the effect of season. Replicates of air sample should be taken at each sampling area to evaluate various detecting methods and to increase the data accuracy.

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