THESIS

EVALUATIONS THAT INCREASE VALUE FOR PORK EXPORT PRODUCTS

Submitted by

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ABSTRACT

EVALUATIONS THAT INCREASE VALUE FOR PORK EXPORT PRODUCTS

Experiment 1: An evaluation of the suitability of porcine lung tissue for human consumption

This study was conducted to provide evidence of the safety of pork lungs for human consumption via an assessment of prevalence of potentially pathogenic bacteria and infectious agents. Specifically, the goal was to collect evidence that could be used to petition the current regulation disallowing use of pork lungs for human food. Pork lungs have been labeled by the U.S. Meat Export Federation as a widely consumed product across Asia as well as South and Central America. It was believed that there is profit potential in saving pork lungs and exporting them to specified countries. Pork lungs must first be deemed safe and edible before they can be sold on the export market. Lungs (N = 288) were collected from a total of six federally inspected young market barrow/gilt or sow processing facilities. In an attempt to obtain a representative sample of production at each facility on a given day, lungs were randomly selected throughout the entire production day. All collected lungs were removed and processed using aseptic techniques to prevent any exogenous contamination. Lung samples were tested for the presence of pathogens and other physical contamination. Lungs did not test positive for *Yersinia* spp., *Influenza,* or *Mycobacterium* spp., and they contained low yeast and mold counts. However, multiple lung samples collected from both barrows/gilts and mature sows tested positive for *Salmonella* spp., Shiga toxin-producing *Escherichia coli*, *Campylobacter,* and *Streptococcus suis*. Also, half of the samples collected were found to contain aspirated plant material within the

airways of the lungs. These results suggested that pork lungs are not safe and should not be saved for human consumption.

Experiment 2: Pork Fibrin used as a meat binder in pork variety and offal meats

Fibrin is a cold set binding product that is created by recombining the two blood components of thrombin and fibrinogen. This study was conducted as a proof-of-concept to validate using fibrin derived from pork blood to create value-added export items from various pork offal and variety meats, hence adding value to both pork blood and pork offal/variety meats. Fibrin currently is marketed as Fibrimex® by Sonac, but the patent for producing fibrin expired leaving potential for U.S. pork operations to begin to produce their own fibrin and use it to create their own value-added products. A total of eight finished products were created in this study using Fibrimex® and pork offal/variety meats. Products for which use of the fibrin complex proved useful included a boneless baby back rib-like product made from pork jowl, a steak-like product made from diaphragms, a boneless hock, a log of skinned pork tongues, a pinwheel with pork diaphragm and cheese, a steak made from course ground heart and back fat, fresh bacon made from pork jowl, and a bung roll stuffed with liver, heart, and kidneys. These products were examples that demonstrated the binding capabilities of fibrin on offal/variety meats that differed in texture. All products were believed to have potential as successful export items. It was noted that fibrin could be added to many other meats to create additional products, including products that are of value within our country's own markets.

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CHAPTER I

INTRODUCTION

Global pork trade, as expressed in dollar value, declined from 2014-2016, yet, U.S. pork exports have continued to increase over this period (USDA-FAS, 2015). The U.S. pork industry exported approximately 1.9 million tonnes of product in 2014; almost twice the amount exported in 2005 (1 million tonnes; USMEF, 2015). North American pork exports are projected to continue to grow and lead all other nations over the next ten years (OECD-FAO, 2014). However, the U.S. pork industry will need to continue to develop innovative ways to market their products in order to continue their impressive export growth rate. The U.S. pork industry can seek a competitive edge in exports by including new value-added products that are marketable in foreign countries through research conducted to determine ways to utilize products that are currently condemned at the time of slaughter.

By-products such as lungs, livers, brains, kidneys, blood, spleens, and tripe have a high nutritive value and are a major component of the diet in many foreign countries (Nollet and Toldra, 2011). Pork blood is a product that has been minimally utilized for edible purposes by slaughterhouses, with the majority of blood being converted to blood meal or dried for fertilizer. Blood has long been used as an ingredient in many foods such as blood sausages, soups, puddings, breads and crackers. Blood collected from slaughterhouses can be added to these products directly or be converted to other products for food as well. The primary purpose for adding blood or blood constituents to meat based products is to increase protein levels and enhance the water binding capabilities of that product (Mandal et al., 1999).

One product in particular, fibrin, is procured from blood components fibrinogen and thrombin and acts as a binder in restructured meat products. Fibrin acts a cold-set binding product that can be used with fresh meat. The first component is fibrinogen, which is a protein in blood, and the other component is thrombin, an enzyme from blood that acticates the binding process (Ryan et al., 1999; Barrett et al., 2004; Toldra et al., 2012). Fibrin naturally occurs when the protein fibrinogen is activated by the enzyme thrombin, thereby forming a strong bond of muscle tissue. Fibrin is effectively used in the meat and food industries to create value-added muscle foods. Fibrin currently is sold commercially in the U.S. as Fibrimex® by Sonac, a European company owned by Darling International. The patent held by Sonac for producing fibrin from blood has expired (Paardekooper and Wijngaards, 1986); therefore, it is reasonable to believe that U.S. pork operations could begin to collect blood at the time of slaughter and isolate fibrinogen and thrombin from blood in house to create fibrin. Ultimately, fibrin has potential as a binding agent to create novel items for export markets using variety meats and offal items.

Lungs are another potential export item that remain relatively unexplored from an edibility standpoint. Results from a brief email survey to U.S. Meat Export Federation Regional Directors indicated that domestic and imported porcine and bovine lungs were consumed by humans in Asia as well as South and Central America. With the majority of international markets consuming porcine lungs, an enormous opportunity for exporting lungs into those markets may exist. However, United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) regulations prohibit saving lungs from livestock for the purpose of human food. According to USDA-FSIS regulation, 9 CFR 310.16, livestock lungs "shall not be saved for human food". This regulation became a final rule on June 17, 1971, and seemingly has not been disputed or explained since. In a separate document for "proposed rule making" dated December

31, 1969, further explanation of the reasoning behind not allowing lungs from livestock for human consumption was explained briefly. Other than the information provided in the aforementioned document, there is minimal, if any, explanation for deeming lungs inedible for humans. Specifically for pork, production practices have changed drastically over the past 40 years and an investigation of the suitability of lungs for human consumption was warranted.

CHAPTER II

REVIEW OF LITERATURE

Fibrin

Binders

Meat binders have been used for many years to create innovative restructured products. Examples of products in which binding technologies are used include sectioned and formed hams, deli meats, sausages, emulsified meat products, and restructured intermediate value products. Products containing binders have grown in popularity due to variety, consistency of quality, convenience, and the economic preference for manufacturers to upgrade low-value raw meat cuts to higher-value products (Sheard, 2002). Binders help to create value-added meat products from valueless meat pieces or trim, and the capability of retaining moisture to increase yields makes them a technology that meat processors are becoming more attracted to and interested in given today's high meat prices (Tsai et al., 1998).

Binders that are added to meat products all have their own unique purpose and binding matrix which takes into account inclusion levels, pH, temperature, and type of meat they are added to. The two categories of binding agents include those that enhance the binding of pieces of meat and those that improve the water-binding capacity of the final product (Pearson and Gillett, 1996). Within these two categories of binding agents, there are two primary types of binders; thermal-set (hot-set) and chemical-set (cold-set) binders (Boles and Shand, 1999).

Salt and phosphate are commonly used to extract salt-soluble proteins in restructured meat products to form emulsions and increase both the binding capability and water holding capacity of pieces of meat by thermally binding myofibrillar proteins that are extracted using an

agitation process such as massaging or tumbling (Schmidt and Trout, 1982; Pearson and Gillett, 1996). Products using salt and phosphates to create a binder are primarily sold as ready-to-eat products (Boles and Shand, 1999). Other binders include raw egg whites, dried egg albumen, sodium caseinate, dried milk, soy protein, and food grade blood proteins; all bind via gel formation rather than by an extraction of salt-soluble myofibrillar proteins among meat pieces (Pearson and Gillett, 1996). Research reported here primarily focused on a cold-set binder referred to as fibrin, which uses blood proteins and enzymes that have gelation properties to enhance binding of meat to form restructured raw meat products (Ofori and Hsieh, 2011).

Fibrin Formation

Fibrin is a cold-set binding agent that currently is marketed under the trademark name of Fibrimex® (Sonac). Fibrin is made by combining thrombin and fibrinogen, which are components of blood often isolated aseptically from cattle or swine at the time of slaughter (Toldra et al., 2012). Thrombin is a serine proteinase enzyme that is the final component of blood coagulation (Barrett et al., 2004). Fibrinogen is a blood protein that acts as the substrate for thrombin, and when combined together, the product fibrin is formed (Ryan et al., 1999).

The methodology for obtaining the two components was developed by the expired fibrin patent developers Paardekooper and Wijngaards (1986). Blood that is collected to retrieve thrombin and fibrinogen is initially stored at 4°C and then separated into blood cells and plasma. Plasma contains the thrombin which is separated by ion-exchange chromatography. A solution consisting of 0.2 M NaCl in 10mM of sodium citrate removes the unbound proteins. Remaining prothrombin is diluted 20 times in water and then activated by a meat thromboplastin from a meat extract in 0.6 M of calcium chloride. Final thrombin product used to make fibrin is frozen immediately and contains 20 NIH-U/ml of thrombin, 0.05 M NaCl, 0.5 mM sodium citrate, and

0.6M $CaCl_2$. Fibrinogen also is collected from blood plasma; but, plasma is first frozen at -3°C and then melted to 0°C before fibrinogen can be separated via centrifuge. Partially-purified fibrinogen is then bagged and frozen immediately.

Fibrin Application

Fibrinogen and thrombin must remain frozen until they are used. Sealed containers containing the thrombin and fibrinogen are submerged in a 27°C water bath until completely thawed (Boles and Shand, 1999). A thrombin:fibrinogen ratio between 1:20 to 1:10 is added to the meat at an inclusion rate between 5% and 10% to initiate the binding process (Scientific Panel on Food Additives, Flavourings, Processing Aids, and Materials in Contact with Food, 2005). The percentage of fibrin added to the meat largely depends on particle size. An increased surface area requires a greater percentage of fibrin (Paardekooper and Wijngaards, 1986). Fibrin forms cross-links with collagen which enables binding. Cross-linking occurs within 10-15 minutes after addition and, therefore, must be mixed quickly (Boles, 2011). Binding occurs best when pH of meat is around 7.0 and the direction of the meat fibers run parallel (Boles and Shand, 1998; Chen and Lin, 2002). Binding of formed product improves when an immediate vacuum is drawn to reduce air pockets that could prevent proper binding from occurring between meat pieces (Paardekooper and Wijngaards, 1986). Final product is then chilled at approximately 0°C for a minimum of 5 hours to allow bonding to proceed (Lennon et al., 2010).

Fibrin Chemistry

A fibrin polymer results from the thrombin and fibrinogen reaction and is the primary clotting factor in whole blood (Weisel and Litvinov, 2013). A multiple-step reaction between fibrinogen and thrombin is required for the fibrin polymer to form which enables binding of meat. Fibrinogen is made up of two identical halves that each contain three distinct peptide

chains (Aα, Bβ, and γ) held together by disulfide bonds (Ryan et al., 1999). Thrombin is a plasma enzyme necessary for formation of fibrin. Thrombin cleaves the A and B fibrinopeptides from the fibrinogen and exposes the binding sites, called A and B, converting the molecule to fibrin (Mosesson, 2005; Rosenfeld et al., 2015). The binding sites interact with the ends of other fibrin molecules and the enzyme transglutaminase (found in the partially-purified fibrinogen) cross-links fibrin monomers to assemble in a half-staggered manner into two-stranded protofibrils. Fibrils then continue aggregating to form fibers that reach out into a three-dimensional network. The fibrin network then cross-links with collagen to complete the binding process (Ryan et al., 1999; Lennon et al., 2010).

Food Safety – Fibrin

The USDA-FSIS currently lists beef fibrin as a safe and acceptable food additive in the *Food Standards and Labeling Policy Book* (USDA-FSIS, 2005). Thrombin and fibrinogen are deemed safe because they are derived from edible animal parts. Blood collected from livestock that is to be used for human consumption must be collected aseptically and pass proper inspection protocols. There have been no known indications that blood fibrin added to food has an impact on allergic or intolerance response after consumption (Scientific Panel on Food Additives, Flavourings, Processing Aids, and Materials in Contact with Food, 2005). Fibrin is degraded by proteases and consequently should be degraded by the intestinal enzymes when consumed (Kolev et al., 1996; 1997). Thrombin is very unstable at high temperatures and low pH conditions and is therefore essentially eliminated from the final product after cooking and consumption (Le Borgne and Graber, 1994).

Effects of Fibrin on Meat Quality

Binders added to meat products have potential of changing slicing characteristics, flavor, color, protein levels, yield, and overall eating quality of meat products (Pearson and Gillett, 1996). Each binding technology works differently based on ingredients and condition of the meat (Boles and Shand, 1999). In general, cold-set binders have been found to reduce issues regarding meat color and oxidative rancidity (Means and Schmidt, 1987; Raharjo et al., 1989). Cold-set bound products are often times more consumer-friendly because they can be utilized in a more versatile manner since they are often similar to fresh cuts of the same size (Esguerra, 1994).

Addition of fibrin to meat products has multiple effects. Boles and Shand (1999) reported differences in color, multiple processing parameters, protein content, and sensory ratings when steakettes made from the two cold-set binders alginate and Fibrimex® were compared. Steakettes manufactured with Fibrimex® had lower protein content and higher moisture content than steakettes made using alginate as a binder, which was expected given the fact that Fibrimex® is about 85% moisture. Consequently, steakettes bound by Fibrimex® were lower yielding, which also was observed by others (Chen and Trout, 1991; Esguerra, 1994; Boles and Shand, 1998). Steakettes made using Fibrimex® also had a higher bind strength and greater dimensional changes during cooking than steakettes made with alginate. These results differed from those of Esguerra (1994), who found that steakettes made from Fibrimex® had lower cooked bind strength. Steakettes manufactured using Fibrimex® were found to be redder in color than those bound with alginate. Finally, sensory panel ratings suggested that consumers found differences in juiciness, texture, and overall acceptability of Fibrimex® steakettes made from different cuts, but not for steakettes made using alginate as a binder.

Summary

Pork fibrin has the gelation properties necessary to enhance binding of pieces of meat to form restructured raw meat products (Ofori and Hsieh, 2011). Aseptic collection of pork blood opens up the opportunity for it to be centrifuged and separated into fibrinogen and thrombin, which can be combined to form fibrin (Paardekooper and Wijngaards, 1986; Toldra et al., 2012). Binding occurs when the fibrin network generated by the reaction between fibrinogen and thrombin cross-links with collagen of meat (Ryan et al., 1999; Lennon et al., 2010). Meat pieces can be successfully bound using a thrombin:fibrinogen ratio between 1:20 to 1:10 and an inclusion rate between 5% and 10% (Scientific Panel on Food Additives, Flavourings, Processing Aids, and Materials in Contact with Food, 2005). Fibrin was declared to be a safe and acceptable food additive by USDA-FSIS in the *Food Standards and Labeling Policy Book* (USDA-FSIS, 2005).

Pork Lungs

USDA Regulations on Livestock Lungs

In 1971, the USDA-FSIS declared that livestock lungs cannot be saved or used as human food (9 CFR 310.16). At this point, livestock lungs that are not condemned by USDA may be used in pet foods or other nonhuman foods (Post-Mortem Inspection, 1971). There has been very little research conducted addressing why lungs are considered inedible and what, if any, pathogens or debris reside within lung tissue. Lungs currently are consumed in foreign countries, which presents an opportunity to conduct more research on livestock lungs in order to determine whether or not the current regulation that prevents them from being saved for human food in the U.S is permitted.

Salmonella spp.

The Centers for Disease Control and Prevention (CDC) estimated that approximately 1.2 million illnesses and 450 deaths occur annually in the U.S from salmonellosis caused by *Salmonella* spp. (CDC, 2015a). *Salmonella* spp. are recognized as one of the most common causes of foodborne illness in humans, however, most cases caused by farm stock are associated with poultry (Rajic and Keenliside, 2001; Callaway et al., 2008). An estimated 8% of *Salmonella* cases in humans are associated with contaminated pork and pork products (IFSAC, 2015). *Salmonella* spp. have been found in the entire digestive track, lymphatic tissue, and stomach of swine (Mogstad, 1995). One study showed that 16 different *Salmonella* serotypes were found in fecal material of swine collected in the slaughter plant (Currier et al., 1986). The fact that *Salmonella* spp. are prevalent in swine makes it a critical pathogen that needs to be considered in research associated with pork products.

Salmonella is considered a motile, non-sporeforming, Gram-negative, rod shaped bacteria that is found most readily in intestinal tracts of a host (Watson et al., 1995; Coburn et al., 2007; FDA, 2012a). The infective dose of *Salmonella* can be as few as one cell and it can cause either nontyphoidal salmonellosis or typhoid fever (FDA, 2012a). Salmonellosis is typically caused by oral ingestion of an infected product. Once consumed, *Salmonella* cells will pass from the intestinal tract into the epithelium, cause inflammation, and release potent enterotoxins and endotoxins (Aberlene et al., 2012; FDA 2012a). Symptoms of nontyphoidal salmonellosis usually last 4-7 days and include nausea, vomiting, abdominal cramps, diarrhea, fever, and headache (FDA, 2012a). Symptoms of typhoid fever will last 2-4 weeks and include high fever, abdominal pains, diarrhea, headache, loss of appetite, and red spots on the skin (FDA, 2012a).

Escherichia coli O157:H7/non-O157 STEC

Escherichia coli are bacteria often found in the digestive tract microflora of animals (CDC, 2015b). Most *E. coli* bacteria are harmless to humans, however, *E. coli* O157:H7 and six Shiga toxin-producing *E. coli* non-O157 serotypes (O26, O45, O103, O111, O121, and O145) are considered to be harmful pathogenic bacteria to humans (CDC, 2015b). The types of STEC found in different species varies. Harmful STEC has been found in swine excrement, including O26, O111, and O121 (Rios et al., 1999; Fratamico et al., 2004; Zweifel et al., 2006; Beutin et al., 2008). Swine are arguably the most similar to humans out of primary livestock species since they are monogastrics, and they tend to be more prone to disease caused by STEC (Bertschinger and Gyles, 1994; Gyles, 2007). The CDC calls *E. coli* O157:H7 one of the most common foodborne pathogens that causes illness in humans (CDC, 2015b). The *E. coli* O157:H7 outbreak that occurred from undercooked beef patties at Jack-in-the-Box restaurants in 1993 was perhaps the most famous food outbreak that the meat industry ever experienced and was the primary reason why it is the most concerning pathogen for all protein producers and packers (CDC, 1993). Prevalence of *E. coli* O157:H7 in swine wasn't actually documented in swine in the U.S. until 2003 (Feder et al., 2003). There is still limited research regarding how prevalent the six primary STEC and *Escherichia coli* O157:H7 are in swine products, including lungs.

E. coli O157:H7 is classified as enterohemorrhagic *Escherichia coli* (EHEC). These serotypes of *E. coli* are Gram-negative, rod-shaped bacteria that produce Shiga toxins known to cause illness in humans (Paton and Paton, 1998; Bettelheim, 2003; FDA, 2012a; CDC, 2015b). The STEC route of entry is oral and once ingested, the bacteria attach to intestinal epithelial cells and produce Shiga toxins (stx) that pass into the bloodstream and become systemic (FDA, 2012a). The infective dose for *E. coli* O157:H7 is typically in the range of 10 to 100 cells and the

infective dose for the other six disease causing STEC is considered to be slightly higher (FDA, 2012a). Humans infected by STEC may experience hemorrhagic colitis, which is characterized by severe abdominal pain, nausea, and bloody diarrhea (FDA, 2012a). Hemorrhagic colitis has the potential to progress into haemolytic uremic syndrome (HUS) which occurs when toxin causes endothelial cell degeneration revealed by swelling which leads to thrombosis and acute renal failure (Tarr et al., 2005; Aberlene et al., 2012). Young children and elderly people are the most prone to developing HUS (Tarr et al., 2005; CDC, 2015b).

Campylobacter spp.

Campylobacter spp. are found in the intestinal tract of animals and cause campylobacteriosis in humans (Altekruse et al., 1999; FDA, 2012a; CDC, 2015c). *Campylobacter* spp. have been estimated to cause 845,000 illnesses annually, which makes it the fourth most prevalent pathogen that contributes to human illness in the U.S. (CDC, 2011). Campylobacteriosis is most commonly associated with *Campylobacter jejuni*, which accounts for approximately 80% of reported cases (FDA, 2012a). Although *Campylobacter jejuni* is the most common *Campylobacter* cause of illness in humans, *Campylobacter coli* is the main species that has been isolated in swine and it presents the challenge of being more resistant to antimicrobial interventions (Bywater et al., 2004; Englen et al., 2005). Multiple studies on swine have shown that *Campylobacter coli* is much more prevalent in the gut and on the carcass of swine than *Campylobacter jejuni* (Mafu et al., 1989; Pearce et al., 2003; Farzan et al., 2009; Abley et al., 2011). Also, survival of *Campylobacter jejuni* outside of the host is poor when compared to *Campylobacter jejuni* (Ketley, 1997). Studies conducted on *Campylobacter* spp. in swine were encouraging from a public health point of view because *Campylobacter coli* was found to be the primary species in pork and not *Campylobacter jejuni*.

Campylobacter spp. are motile, spiral-shaped, non-sporeforming, and Gram-negative bacteria that thrive in warm environments with oxygen levels ranging from 3% to 5% (FDA, 2012a; CDC, 2015c). Most *Campylobacter* spp. are fairly fragile and sensitive to freezing, drying, acidic conditions, and salinity (FDA, 2012a; CDC, 2015c). The pathogenesis of *Campylobacter* is fairly unknown, but research shows that if ingested, *Campylobacter* cells will cause infection by invading and colonizing the gastrointestinal tract (FDA, 2012a). The minimum infective dose of *Campylobacter* is estimated to be about 10,000 cells (FDA, 2012a). Humans who have been infected with *Campylobacter* have the possibility of contracting campylobacteriosis, or *Campylobacter* enteritis, which includes symptoms of fever, diarrhea, cramps, and vomiting that typically lasts two to ten days (FDA, 2012a). Children younger than 5 years old and young adults 15 to 19 years old are the most prone to contracting campylobacteriosis (FDA, 2012a; CDC, 2015c).

Yersinia spp.

Swine are a primary reservoir for *Yersinia* spp. that cause yersiniosis in humans (Funk et al., 1998). Undercooked pork that has been contaminated with *Yersinia* spp. is typically the source for humans who contract yersiniosis (FDA, 2012a). *Yersinia* spp. are primarily found in the lymph nodes but are also prevalent in the caecal content, tongues, and oral-pharyngeal fluid of swine (Lee et al., 1990; Bhaduri et al., 1997; Funk et al., 1998; Pujol and Bliska, 2004). Specific locations that *Yersinia* spp. are found within the animal makes the entire carcass prone to contamination if necessary processing techniques and interventions aren't used appropriately. The three *Yersinia* spp. that are known to cause disease in humans are *Yersinia enterocolitica, Yersinia pseudotuberculosis,* and *Yersinia pestis*, however only the first two species mentioned are prevalent in swine (Funk et al., 1998; Pujol and Bliska, 2004; Bowman et al., 2007).

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are small, rod-shaped, Gram-negative bacteria that have the ability to grow below 4°C and can survive freezing temperatures (FDA, 2012a). The average infective dose of pathogenic *Yersinia* spp. is believed to be between 10^4 and 10^6 organisms (FDA, 2012a). *Yersinia* spp. that have infected a host, establish colonies within the lymphoid tissue, resist phagocytosis by neutrophils, and ultimately cause cytotoxic changes within the human cells (Pujol and Bliska, 2004; FDA, 2012a). Humans who have ingested *Yersinia* spp. are at risk for developing yersiniosis which causes diarrhea, fever, abdominal pain, and vomiting that lasts anywhere from three days to multiple weeks (FDA, 2012a). Children under the age of 5 are the most prone to yersiniosis (FDA, 2012a).

Staphylococcus aureus

The CDC reported approximately 241,000 foodborne illnesses were caused by staphylococcal food poising, which ranks *Staphylococcus aureus* as the fifth most prevalent pathogen contributing to foodborne illnesses in the United States (CDC, 2011). The pork industry has continually monitored and fought *Staphylococcus aureus* because ham and other pork products are frequently identified as the primary source of staphylococcal food poisoning (Bryan, 1988). Recently, the pork industry has focused specifically on methicillin-resistant *Staphylococcus aureus* (MRSA) (de Boer et al., 2009; Smith et al., 2009; Weese et al., 2010). The MRSA bacteria are resistant to methicillin which is commonly used to treat staphylococcal illness (de Boer et al., 2009). In an EU study conducted by de Neeling et al. (2007) a high prevalence of MRSA was found in pigs from farms and cross contamination occurred within the slaughterhouse. Research shows that swine are premium reservoirs for *Staphylococcus aureus* and MRSA which makes pork products extremely susceptible carriers for the staphylococcal toxin.

Staphylococcus spp. are Gram-positive, non-motile, spherical, and catalase-positive bacteria that produce a highly heat-stable enterotoxin (FDA, 2012a). The enterotoxins produced by the bacteria can cause gastroenteritis, staphylococcal food poisoning, toxic shock syndrome, pneumonia, postoperative wound infection, and nosocomial bacteremia in humans (FDA, 2012a; CDC, 2015d). *Staphylococcus* spp. are very stable bacteria that can grow and survive extended periods in a dry state, in the temperature range of 7°C to 47.8°C, and in a pH range between 4.5 and 9.3 (FDA, 2012a). The toxic dose is typically less than 1 microgram of enterotoxins, which is typically found in foods that exceed 100,000 *Staphylococcal aureus* organisms per gram (FDA, 2012a). Symptoms of staphylococcal food poisoning can develop within an hour after consumption and typically last less than a day (FDA, 2012a; CDC, 2015d). Anyone who consumes an infective dose of staphylococcal enterotoxins is at risk of experiencing the symptoms of staphylococcal food poisoning (FDA, 2012a).

Streptococcus suis

Streptococcus suis is one of the most critical swine pathogens in the world. Research regarding *Streptococcus suis* has recently increased dramatically after a major outbreak occurred in China in 2005 where 204 people were infected and 38 of them died (Lun et al., 2007; Gottschalk et al., 2007; Fittipaldi et al., 2012). A total of 35 serotypes of *Streptococcus suis* have been discovered, however, serotype 2 is considered to be the most virulent and frequently isolated in both swine and humans (Gottschalk et al., 2007; Lun et al., 2007). Pathogenic *Streptococcus suis* is primarily isolated in the upper respiratory tract of infected swine, particularly the tonsils and nasal cavities (Higgins and Gottschalk, 1999). It also is important to note that *Streptococcus suis* is almost constantly present in the lungs of swine, but current regulations prevent the lungs from being saved for human consumption (Gottschalk and Segura,

2000). Recent research regarding *Streptococcus suis* in swine, especially results discovered by Gottschalk and Segura (2000), appeared to show that presence of *Streptococcus suis* in pork lungs is inevitable.

Streptococcus suis is a Gram-positive facultative anaerobe that is coccoid shaped (Lun et al., 2007; FDA, 2012a). The organism is very stable in both wet and dry environments at 0°C and it is able to grow in either aerobic or anaerobic conditions (Lun et al., 2007). *Streptococcus suis* is a zoonotic bacteria that can be transmitted to humans who come in close contact with infected swine or those who consume undercooked infected pork products (Lun et al., 2007; Gottschalk et al., 2007; Fittipaldi et al., 2012). Streptococcal infection occurs when *Streptococcus suis* colonizes the host, penetrates epithelial cells, survives in the bloodstream, and finally invades organs causing inflammation (Fittipaldi et al., 2012). Humans who experience streptococcal infections are likely to develop meningitis, but there have been cases of septic shock leading to organ failure, endocarditis, pneumonia, peritonitis, and arthritis (Lun et al., 2007).

Mycobacterium spp.

Certain *Mycobacterium* spp. have been known to cause tuberculosis, which is responsible for approximately 1.7 million deaths annually throughout the world (CSIS, 2015). Fortunately, tuberculosis is rare in the U.S. and there are only an estimated 60 cases annually, primarily caused by unpasteurized milk (FDA, 2012a). Swine, however, are a primary host species in many other countries where tuberculosis is one of the deadliest infectious diseases (Bolin et al., 1997; Straw et al., 2006; Alvarez et al., 2010; Lara et al., 2011). Swine that are infected with *Mycobacterium* spp. and that have developed tuberculosis generally develop granulomas in the lungs and lymph nodes (Bolin et al., 1997). The primary species groups responsible for causing disease belong to the *Mycobacterium tuberculosis* complex and the *Mycobacterium avium*

complex (Muwonge, 2012). The *Mycobacterium tuberculosis* complex includes species, such as *Mycobacterium bovis,* that are considered highly pathogenic and can cause tuberculosis (Muwonge, 2012). The *Mycobacterium avium* complex includes species that are typically non-pathogenic and do not cause tuberculosis in humans, but can cause pulmonary disease, lymphadenitis, or infections in immunocompromised people (Muwonge, 2012). Both the *Mycobacterium avium* complex and the *Mycobacterium tuberculosis* complex are prevalent in swine raised in many foreign countries.

Mycobacterium spp. are Gram-positive, aerobic, non-motile, rod-shaped bacteria that lack an outer cell membrane (FDA, 2012a). *Mycobacterium* spp. have unique cell walls that enable them to survive exposure to caustic cleaners and resist lysis by antibiotics (FDA, 2012a). The organism is slow growing and most commonly found in water, soil, and bedding (FDA, 2012a). The organism is often found within the lungs, lymph nodes, and gastrointestinal tracts of livestock and humans who are infected with *Mycobacterium* spp. (FDA, 2012a). The toxic dose for *Mycobacterium tuberculosis* is estimated to be less than 10 bacilli, but the toxic dose for *Mycobacterium avium* is unknown. Humans who have been infected with tuberculosis causing *Mycobacterium* spp. generally do not show symptoms until months after the initial infection (FDA, 2012a; CDC, 2015e). Everyone is susceptible to infection from species belonging to the *Mycobacterium tuberculosis* complex and generally only people who are severely immunocompromised are at risk of developing infection from organisms belonging to the *Mycobacterium avium* complex (FDA, 2012a).

Type A Influenza H1N1 (Swine Flu)

The influenza A (H1N1) virus outbreak that began in 2009 is considered to be one of the greatest pandemic threats since the outbreak of influenza A (H3N2) that occurred in 1968 (Novel

Swine-Origin Influenza A [H1N1] Virus Investigation Team, 2009). It was estimated that influenza A (H1N1) was responsible for 60.8 million cases, 274,304 hospitalizations, and 12,469 deaths that occurred in the U.S. between April 2009 and April 2010 (Shrestha et al., 2011). Influenza H1N1 is commonly referred to as swine flu because the first case discovered was similar to the virus found in pigs (Myers et al., 2006). Influenza H1N1 also is zoonotic and can be transferred from humans to swine or from swine to humans (Myers et al., 2006). Swine serve as reservoirs for the generation of reassortant viruses that may be more virulent and can transmit easier to humans than the parental influenza A viruses because they are found to be susceptible to infection with avian, swine, and human influenza viruses (Brown, 2000; Liu et al., 2011). It is important to note that consumers cannot contract the influenza H1N1 virus from eating properly cooked pork, which was a costly misperception the pork industry had to face in the wake of the 2009 outbreak.

Swine flu is a respiratory disease caused by influenza A viruses, such as influenza H1N1, which is present in pigs (APHIS, 2013). Influenza A viruses tend to thrive in large confinement operations with limited ventilation (Myers et al., 2006). Swine and humans infected with influenza A show signs of coughing, high fever, difficulty breathing, runny nose, loss of appetite, and immobility (APHIS, 2013). Swine can be vaccinated for swine influenza and it is also highly advised that those who work with swine receive a seasonal vaccination to prevent influenza A virus transmission from swine to humans and vice versa (APHIS, 2013).

Summary

Pork lungs are currently not allowed to be saved for human consumption based on the USDA-FSIS regulation, 9 CFR 310.16 (1971). Schwabe (1979) along with others have noted that lungs are widely consumed throughout the world. Unfortunately, there is a lack of research

indicating which, if any, pathogens or debris reside within the lungs. Microorganisms of interest to the pork industry include *Campylobacter* spp., *Yersinia* spp., non-O157:H7 STEC's, *Escherichia coli* O157:H7, *Streptococcus suis*, *Mycobacterium* spp., Type A influenza viruses, *Staphylococcus aureus*, and *Salmonella* spp. (Baer et al., 2013). Results showing little to no contamination may serve as defense data to uplift the current ban.

CHAPTER III

AN EVALUATION OF PORCINE LUNG TISSUE FOR HUMAN CONSUMPTION

Introduction

Results from a brief email survey to U.S. Meat Export Federation Regional Directors indicated that domestic and imported porcine and bovine lungs were consumed by humans in Asia as well as South and Central America. European countries proved to be the exception. With the majority of international markets consuming porcine lungs, an enormous opportunity for exporting lungs into those markets could exist. However, USDA-FSIS regulations prohibit saving lungs from all livestock species for the purpose of human food. Specifically, for pork, production practices have changed drastically over the past 40 years. Many of these changes may improve the safety of porcine lungs as a human food.

According to USDA-FSIS regulation, 9 CFR 310.16, livestock lungs shall not be saved for human food. This regulation became a final rule in June 17, 1971, and seemingly has not been disputed or investigated since. In a separate document for "proposed rule making" dated December 31, 1969, further explanation of the reasoning for not allowing lungs from livestock to be used for human consumption was explained briefly. It was specifically stated that several hundred beef lungs were evaluated by trained pathologists, who reported that 93.5% of lungs were affected with various abnormal conditions. This included lungs being adulterated with airborne or induced external substances such as dust, molds, rumen ingesta, nasal exudate, etc. It was determined that these contaminants were imbedded deeply in the smallest "air tubes" (alveoli) of the lungs and that it is not feasible to microscopically examine all parts of the lung before passing them for human consumption. As a result, in 1971, lungs from all livestock

species were no longer permissible for human consumption. Other than the information provided in the aforementioned documents, there is minimal, if any, other explanation for deeming lungs inedible for humans. Interestingly, this is not a mandate in the Federal Meat Inspection Act, and this regulation can be amended or suspended via a formal petition to USDA-FSIS, provided that ample evidence is provided supporting the amendment. Therefore, it was the objective of the proposed research to provide evidence of the safety of porcine lungs for human consumption via determining prevalence of potentially pathogenic bacteria and infectious agents known to be prevalent in pork. This included determining prevalence the seven predominant STEC's, *Salmonella* spp., and *Campylobacter* spp. Additionally, discussions with USDA indicated that pneumonia, and particularly tuberculosis, may be of great concern to USDA-FSIS when considering allowing lungs for human consumption. According to the National Veterinary Services Laboratory (NVSL), who performs the surveillance of tuberculosis for USDA and the Department of Wildlife, *M. bovis* has the greatest potential to be discovered in porcine lungs due to comingling with cattle; however, detecting any of the *Mycobacterium* spp. in domesticated porcine lungs was unlikely due to modern production practices, specifically confinement production practices. *Mycobacterium* spp. are generally known to result from the soil and non-potable water sources, and the vast majority of market hogs (young and old) are not exposed to either of these sources. The supervisor of bacteriology at NVSL, the individual in charge of USDA's national surveillance for tuberculosis, indicated via personal communication that there is not an extremely high likelihood that *Mycobacterium* spp. associated with tuberculosis will be cultured from the lungs of domestic swine. Additionally, most all sources, including USDA, agreed that foodborne tuberculosis is not the primary transmission mechanism in the U.S. and

over 90% of healthy youth and adults are immune; however, exposure to animals infected with *M.bovis* and *M. tuberculosis* can cause paratuberculosis in humans.

Materials and Methods

Pork Lung Collection Process

Lungs (N = 288) were collected in April and May of 2014 from a total of six federally inspected pork processing facilities; four commercial pork processing plants that were harvesting youthful, market weight gilts and barrows and two commercial pork processing plants that were harvesting mature sows. An equal number of lungs (n=48) was collected from each plant. It should be noted that the processing plants harvesting sows were not using a hot water scalding technique to dress the animals. The sows that were harvested were skinned hot on the harvest floor immediately after being bled, whereas the young barrows and gilts were scalded.

In an attempt to obtain a representative sample of the production facility on an average working day, animals and corresponding lungs were randomly selected throughout the entire production day. Pork lungs collected from each of these plants were federally inspected and only lungs that passed inspection were used in the study. In the event that an individual animal or any of its internal organs were condemned, the lungs were not collected from that animal. All of the lungs collected were removed and processed using aseptic techniques to prevent any exogenous contamination. Each lung was removed from the carcass using sterile gloves and then placed on a sheet of parchment paper that had been properly sterilized (autoclaved parchment paper individually and aseptically packaged). In addition, the surface underneath the parchment paper was cleaned and sterilized with a 70% alcohol solution between each collection period. A new pair of sterile gloves and parchment paper was used for each sample. Each sample collected for presence of pathogens was a composite sample consisting of five randomly selected lungs that

were consecutively pulled off the production line, except for the 48 samples collected for histopathological examination. The 48 samples collected for histopathological examination each represented one lung and were randomly selected one at a time every hour.

Processing and Shipping the Pooled Samples for Pathogenic Testing

Each pooled sample consisted of approximately 500 g of lung tissue from five animals. Tissue was removed from lungs using sterilized scissors and scalpels. Each individual lung had approximately 100 g of total tissue removed from the apical lobes, middle lobe, diaphragmatic lobes, and the accessory lobe located on the right side of the lung. Unlike histology samples, there was no lymph node tissue included in pooled samples. All excisions were then placed into a single Whirl-Pak bag which was then placed directly into a refrigerated environment. Immediately following a day's collection, samples were placed in a cooler containing ice packs and then shipped overnight to a commercial laboratory. Samples were adequately insulated with newspaper to prevent direct contact with the ice packs. Microbiological samples were screened by an accredited commercial laboratory (Food Safety Net Services, San Antonio TX) for the prevalence of *Salmonella* spp., *Campylobacter* spp., *Staphylococcus aureus*, STEC, *Yersinia* spp., mold, and yeast. Paired samples were also sent to the University of Minnesota Veterinary Diagnostic Lab to be tested for the prevalence of *Streptococcus suis*. Colorado State University Veterinary Diagnostic Laboratories tested for the prevalence of *Mycobacterium* spp. and Influenza. In each case, prior to prevalence screening, individual (pooled) samples were pummeled/homogenized in order to obtain a representative sample.

Processing and Shipping the Histology Samples

Each histology sample (n = 48) consisted of 5 segments from a single lung, which was randomly selected. For each lung designated for histology, samples were obtained in the

processing plant from the following five anatomical locations: 1) cross-section from the middle of the right apical lobe; 2) cross-section from the middle lobe; 3) tip of the accessory lobe; 4) cross-section from the right diaphragmatic lobe; and 5) tracheobronchial lymph node (Figure 3.1). Samples were removed from the lung using sterilized scissors. Samples were then placed into a Whirl-Pak bag, and refrigerated. For shipping, the Whirl-Pak bags containing the histology samples were placed in a cooler containing cold packs and then shipped overnight to the Colorado State University Diagnostic Lab. Samples were adequately insulated with newspaper to prevent direct contact with the cold packs. Colorado State University conducted the histopathological examinations. Tissue were fixed by immersion in a 10% neutral buffered formalin and routinely processed. Five micron sections of each tissue were stained with hematoxylin and eosin for examination.

Pathogen Testing Procedures

Mycobacteria Procedure

Mycobacteria testing was conducted by Colorado State University Veterinary Diagnostic Lab (Fort Collins, CO) following procedures outlined by Fyock and Whitlock (1999; Appendix A). Lung tissue samples were reduced to 2 g and placed in a conical tube containing 35 ml of sterile water. Tube was mixed vigorously and then placed in a vortex for 30 minutes. Tube was left standing at room temperature for at least 30 min after being removed from the vortex. A total of 5 ml of the sample was removed from the top one third of the test tube and placed in a new 50 ml conical tube containing 25 ml of room temperature BHI/HPC (0.75% HPC). The tubes were incubated at 35-37°C for 18-24 hrs. Samples were then centrifuged for 30 minutes at 900 x *g*. Supernatant was removed and the cellular debris pellet was suspended with 1 ml of the antibiotic brew. Tubes were incubated again at 35-37°C overnight. Four test tubes were inoculated at room

temperature with Herrold's Egg Yolk agar (three with mycobactin and one without) with .250 µl of the resuspension. Samples plated on Herrold's Egg Yolk agar were incubated at 37°C in a slanted position with loose caps. The caps were tightened after 1-2 weeks and moved to an upright position in the incubator. Colony counts were recorded every 2 weeks for 16 weeks (Russell, 2012).

APC Procedure

Aerobic Plate Count testing was conducted by Food Safety Net Services (San Antonio, TX) following *3M's* procedure titled "Petrifilm Aerobic Plate Count" (2014; Appendix B). Petrifilm Aerobic Count plates from 3M were used to analyze aerobic bacteria counts. The samples were diluted with Butterfield's Phosphate Buffer in a sterile bag. Samples were mechanically pummeled for 2 min. Appropriate dilutions were plated to enumerate aerobic plate counts. Petrifilms were incubated with the clear side up for 48 hrs ± 3 hrs at 35°C ± 1°C. APC counts were reported in CFU/g.

Salmonella Procedure

Salmonella testing was conducted by FSNS (San Antonio, TX) following *bioMèrieux's* procedure (VIDAS® Easy SLM; Appendix C). A 25 g sample of lung tissue was enriched with 225 ml of broth and then pummeled for 2 min. Sample was then incubated for 16-22 hrs at 35°C ± 1°C. Next, a 0.1 ml sample of the inoculum was added to the VIDAS strip and placed in the VIDAS Heat and Go to warm for 15 min. The VIDAS strips were removed and cooled at room temperature for 10 min. Results were recorded after 45 min.

Yeast and Mold Procedure

Yeast and mold testing was conducted by FSNS (San Antonio, TX) following the procedures outlined in the Bacteriological Analytical Manual (BAM; Tournas et al., 2001;

Appendix D). Each lung tissue sample was divided into 50 g subsets and applied to plates with Dichloran rose bengal chloramphenicol (DRBC) agar. Samples were incubated in the dark at 25°C for five days. Samples were incubated for an additional 48 hrs to allow time for heat or chemically-stressed cells and spores to grow if no growth was detected after the initial five days.

Campylobacter Procedure

Campylobacter testing was conducted by FSNS (San Antonio, TX) following the DuPont™ BAX® System Real Time PCR Assay for *Campylobacter* (2013; Appendix E). A 25 g sample of lung tissue was diluted by a factor of 1:10 in single-strength Bolton broth. A total of 200 µL of lysis reagent and 5 µL of the diluted sample were added to cluster tubes. The cluster tubes were then heated for 20 minutes at 37°C followed by 10 min at 95°C in a dry block heater. Cluster tubes were cooled for 5 min before 30 µL of their content was transferred to PCR tubes in a cooling block. PCR tubes were placed in a PCR cycler for 90 min to receive the final results.

Streptococcus suis Procedure

Testing for *Streptococcus suis* was conducted by the University of Minnesota Veterinary Diagnostic Lab following their SOP titled "*S. suis* Detection PCR" (2014; Appendix F). Microbial DNA was extracted from pork lung tissue and used for a PCR test. The PCR master mix was prepared using Hot StarTaq mixture, JP4 F primer, JP5 R primer, and PCR water. A 2 µl sample of DNA extracted from the sample was added to the master mix along with a 2 µl sample of template DNA. The PCR reaction tubes containing the sample DNA and PCR master mix were placed into a GeneAmp PCR System 9700 Thermalcycler and ran at the appropriate time and temperatures for the *Streptococcus suis* detection PCR program. A 12 µl PCR product from each sample was added to a 1% TAE-agarose gel stained with ethidium bromide and used for Gel Electrophoresis. A gel image for each sample was collected and the detection of

Streptococcus suis was determined based on the presence of a band at approximately 688 base pairs.

Yersinia spp. Procedure

Testing for *Yersinia* spp. was conducted by FSNS (San Antonio, TX) following the *Yersinia* spp. testing procedures outlined in the BAM (Feng and Weagant, 2001; Appendix G). A 25 g sample was enriched with Peptone sorbitol bile broth (PSBB) and homogenized for 30 seconds. Samples were then incubated at 10°C for 10 days. Enrichment broth was removed from the incubator on day 10 and thoroughly mixed. One loop of enrichment was transferred to 0.1 ml of 0.5% KOH in 0.5% saline and mixed for 2-3 sec. One loop of the new mixture was streaked on a MacConkey agar plate. An additional 0.1 ml of enrichment was added to 1 ml of 0.5% saline, mixed for 5-10 sec, and streaked on an additional MacConkey agar plate. Plates were incubated for 1-2 days at 30°C and examined for colonies. No suspected *Yersinia* spp. grew on the MacConkey agar so no additional confirmatory agars were needed.

Staphylococcus aureus Procedure

Testing for *Staphylococcus* aureus was conducted by FSNS (San Antonio, TX) following the *Staphylococcus aureus* testing procedures outlined in the BAM (Bennett and Lancette, 2001; Appendix H). A 1 ml sample was distributed equally to 3 plates of Baird-Parker agar. Inoculum was spread over the surface of agar and placed inverted in an incubator for 45-48 hrs at 35°C. Plates were removed and colony counts were recorded. Samples that did not have any visual growth of *Staphylococcus aureus* were recorded as having < 10 CFU/g.

Non-O157:H7 STEC Procedure

Testing for non-O157:H7 STEC was conducted by FSNS (San Antonio, TX) following the USDA-FSIS Microbiology Laboratory Guidebook 5B.05 (2014; Appendix I). A 325-375 g

sample was placed in a sterile bag with a mesh filter. A total of 975 g of Modified Tryptone Soya Broth (mTSB) was added to the sample and pummeled until well mixed. Samples were incubated at 42°C for 15-24 hrs. Samples were analyzed by a real-time PCR using the BAX® system.

Type A Swine Influenza Virus Procedure

Testing for Type A Influenza was conducted by Colorado State University Veterinary Diagnostic Lab following the National Veterinary Services Laboratory's SOP written by Koster (2012; Appendix J). Real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) SOP for the National Veterinary Services Laboratories was used as a reference for the detection of Type A Swine Influenza Virus. A probe monitored the target PCR product formation at each cycle during the PCR reaction. Probes were labeled with a reporter dye at one end and a non-fluorescing quencher at the other end. The amount of fluorescence that was generated and the cycle number of detection was proportional to the amount of target template.

Results and Discussion

The primary objective of this research was to determine whether or not pork lungs could in fact be saved for human consumption. Microbiological tests were conducted to determine prevalence of key pathogens of interest to the pork industry and we completed a histopathological examination of pork lungs to determine whether or not physical contamination resides within lung tissue. Current USDA-FSIS regulations prevent all livestock lungs from being saved for human consumption within the U.S. Unfortunately, this rule is currently preventing the pork industry from capturing a potential economic benefit if in fact pork lungs are safe to eat. However, results from this project demonstrated that prevalence of certain pathogens,

such as *Salmonella* and *STEC*, and contamination with foreign materials occur at high frequencies in pork lungs at slaughter (Table 3.1).

Pathogen data

Samples collected for specific pathogen testing were pooled samples that each contained lung tissue from five hogs. A total of 49 samples that were retrieved from both the market barrows/gilts and the mature sows tested positive for *Salmonella* spp., Shiga toxin-producing *Escherichia coli*, *Campylobacter,* and *Streptococcus suis*. *Salmonella* and STEC were the most prevalent in the pooled lung samples. *Salmonella* was found in approximately 54.2% of all the samples collected and at least one STEC was found in approximately 31.3% of the samples (Table 3.1). All fifteen of the samples testing positive for STEC contained more than one Shiga toxin-producing *E. coli* and three of these samples actually contained all six of the major STEC (Table 3.2). One sample (2.3%), tested positive for *Campylobacter* (Table 3.1). *Streptococcus suis* was found in 21.9% of the samples from young market hogs but was not found in any of the samples collected from the sows (Table 3.1). Prevalence of *Yersinia* spp*., Influenza,* and *Mycobacterium* spp. were also tested for, however no samples tested positive for these pathogens.

Histology data

Histopathology results indicated that 25 of the 48 samples from the 48 hogs contained aspirated material (Table 3.3). Aspirated material included either plant material, blood, or fluid that appeared to originate from oropharynx. Of the 25 lung tissue samples that showed evidence of aspirated material, 24 of these samples came from hogs that were sent through a hot water scalding process. The remaining sample that contained aspirated material was from a sow that was skinned and not scalded. Figure 3.2 shows that 52.1% of all the hogs sampled aspirated,

75% of the hogs that were hot scalded aspirated, and 6.3% of the hogs that were skinned and not hot scalded aspirated. Although aspiration appeared to be much more common in the hogs that were scalded, prevalence of major pathogens found in these hogs was less than pathogen prevalence in hogs that were skinned. Table 3.3 shows the pathogens prevalent in the lung tissue under the two different processing methods (skinning or hot scalding).

APC, Staphylococcus aureus, Mold, and Yeast Data

In addition to the previously mentioned pathogens that were tested for, mold, yeast, *Staphylococcus aureus,* and aerobic plate counts were also taken from each composite sample. Mold counts for all the samples were not detectable (<10 CFU/g). Yeast counts for lungs from all sows were not detectable (<10 CFU/g), however, 20 of 24 samples from young market hogs had yeast counts >10 CFU/g. *Staphylococcus aureus* was not detectable (<10 CFU/g) in 47 of 48 lung samples, however, it was detected (60 CFU/g) in one sample from a young market hog. Aerobic plate counts were high in lungs from both sows and market hogs. The average APC for the market hog lung samples was 23,838 CFU/g but only 3,115 CFU/g for the sows (Table 3.4).

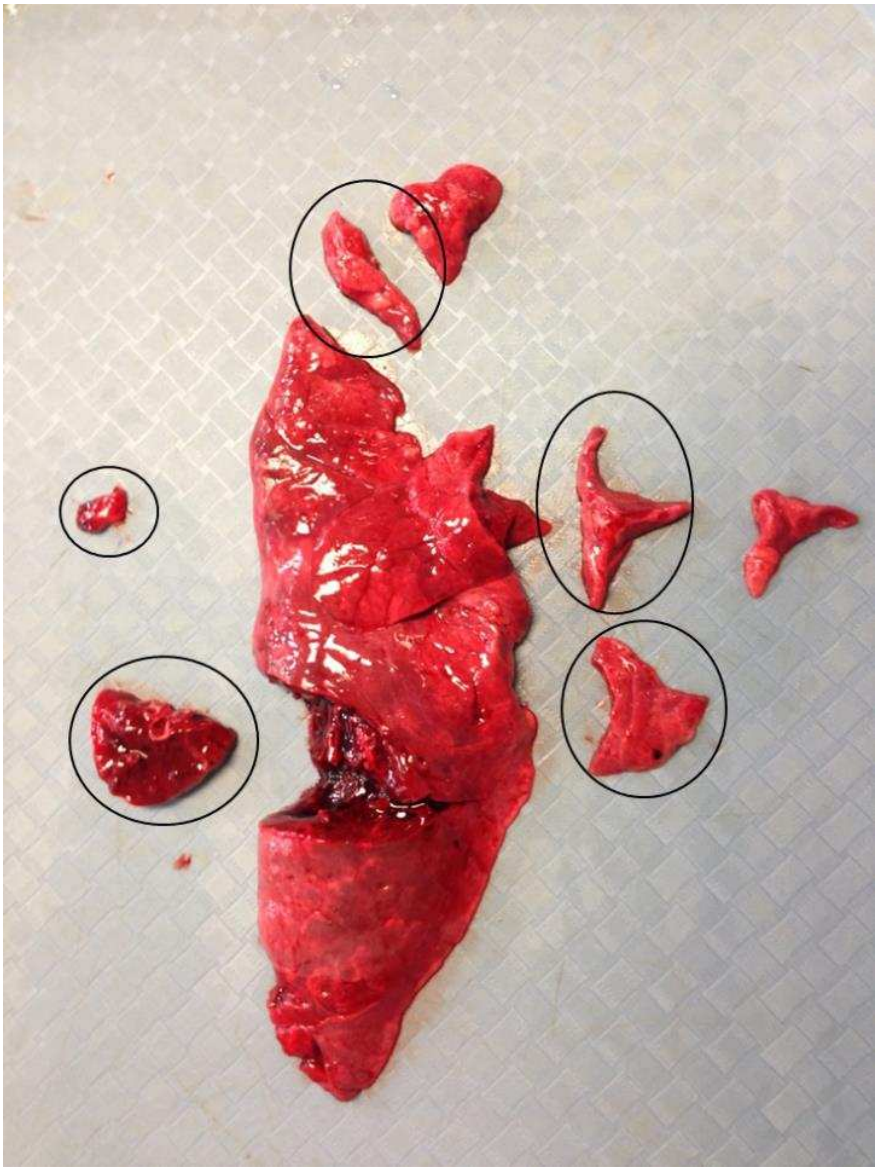

Figure 3.1. Anatomical locations of pork lung histology samples.

Table 3.1. Pathogen prevalence found in pork lungs (n = 48).

Pathogen	Total samples, young[2]	Positives	% Positives	Total samples, mature[3]	Positives	% Positives	Total samples	Total positives	Total % positive
Salmonella	32	16	50.0%	16	10	62.5%	48	26	54.2%
Campylobacter	27	0	0.0%	16	1	6.3%	43	1	2.3%
STEC[1]	32	10	31.3%	16	5	31.3%	48	15	31.3%
Yersinia spp.	32	0	0.0%	16	0	0.0%	48	0	0.0%
Strep. suis	32	7	21.9%	16	0	0.0%	48	7	14.6%
Influenza	32	0	0.0%	16	0	0.0%	48	0	0.0%
Mycobac. spp.	32	0	0.0%	16	0	0.0%	48	0	0.0%

[1]Shiga toxin producing Escherichia coli.
[2]Market ready barrows and gilts.
[3]Sows which have farrowed at least one litter.

Table 3.2. Frequency of individual STEC strains[1] found in pork lungs (n = 48).

	Number of STEC					
	1	2	3	4	5	6
Barrow/gilt samples	0	2	1	1	3	3
Sow samples	0	3	2	0	0	0
Total samples	0	5	3	1	3	3

[1]Shiga toxin producing Escherichia coli strains tested for include O26, O45, O103. O111, O121, and O145.

Table 3.3. Pathogens prevalent in pork lungs using different processing techniques.

Processing Technique	Salmonella	STEC	Campylobacter
Scalding	50.0%	31.3%	0.0%
Skinning	62.5%	31.3%	6.3%

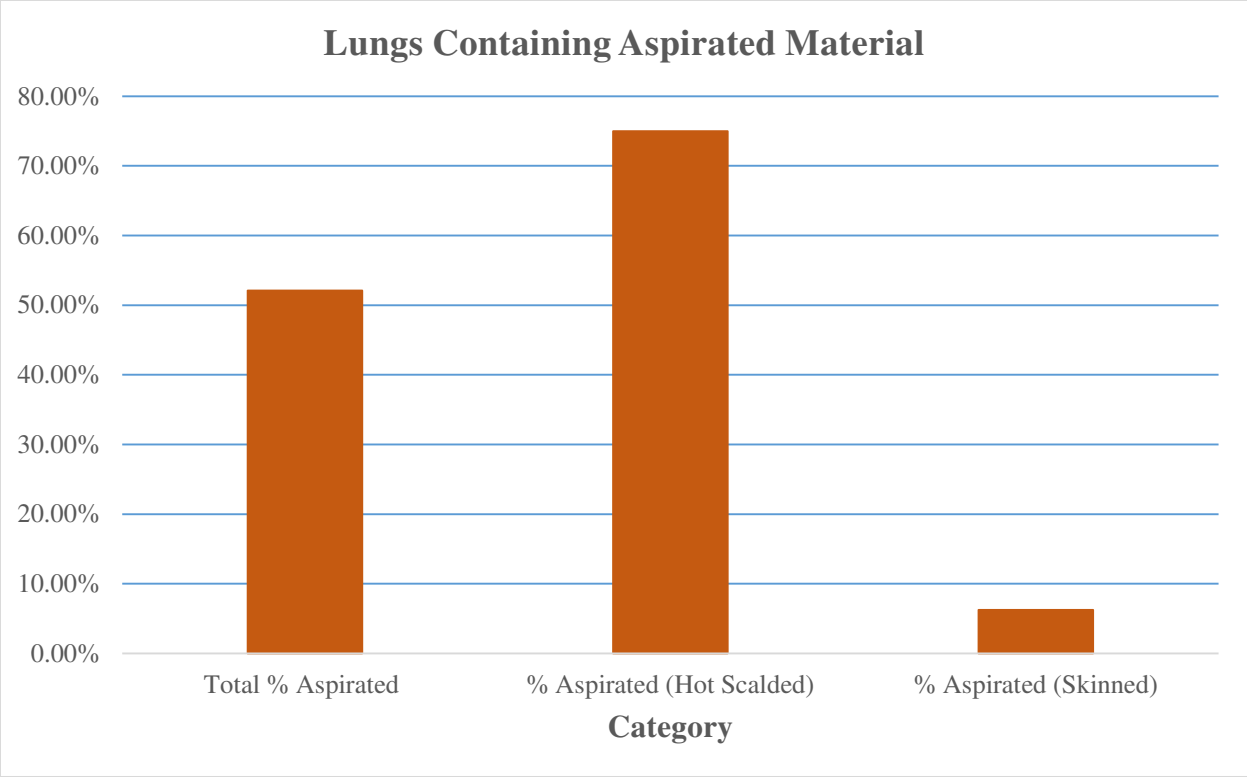

Figure 3.2. Frequency distribution of lungs containing aspirated material among the total hogs, scalded hogs, and skinned hogs (n = 48).

Table 3.4. Mold, yeast, Staphylococcus aureus, and aerobic plate counts (APC) in pooled pork lung samples.

	Mold avg.	Staph. aureus	APC avg.	Yeast avg.
Barrow/Gilt Samples	<10 CFU/g	<10 CFU/g	23,838 CFU/g	255 CFU/g
Sow Samples	<10 CFU/g	<10 CFU/g	3,115 CFU/g	<10 CFU/g
Total Samples	<10 CFU/g	<10 CFU/g	16,930 CFU/g	127 CFU/g

CHAPTER IV

PORK FIBRIN USED AS A MEAT BINDER IN PORK VARIETY AND OFFAL MEATS

Introduction

Binders have been used by the meat industry for many years to create innovative restructured products. Fibrin is an example of one particular cold set binding product that can be used on fresh meat. Fibrin is produced by combining two components extracted from blood. The first component is fibrinogen, the primary binding protein, and the other component is thrombin, an enzyme that catalyzes cross-linking of collagen helices leading to the binding process (Ryan et al., 1999; Barrett et al., 2004; Toldra et al., 2012). Fibrin naturally occurs when the protein fibrinogen is activated by the enzyme thrombin, thereby forming a strong bond of muscle tissue. Fibrin is effectively used in the meat and food industries to create value-added muscle foods. Fibrin is currently sold commercially in the U.S. as Fibrimex® by Sonac, a European company owned by Darling International. The patent held by Sonac for manufacturing fibrin from blood has expired (Paardekooper and Wijngaards, 1986); therefore, it is reasonable to believe that U.S. pork operations could begin to collect blood at the time of slaughter and isolate fibrinogen and thrombin from blood in house to create fibrin. Ultimately, fibrin has potential as a binding agent to create novel items for export markets using variety meats and offal items.

Materials and Methods

A total of eight products were developed using pork variety/offal meats and beef fibrin purchased from Sonac. Beef fibrin was the only fibrin available from Sonac at the time of this project. The commonly exported items that were used are listed in Table 4.1. Fibrin obtained

from Sonac was sold frozen in two separate containers; one containing fibrinogen and the other containing thrombin. The fibrinogen and thrombin remained frozen before use.

Fibrinogen and thrombin containers were submerged in 26.6°C water until they reached liquid form. Initial temperature of the water must be 26.6°C in order to maintain the binding factors of the two components. Fibrinogen and thrombin were mixed together at a ratio of 10:1 to create the fibrin. Binding began when fibrin was added to the protein source. Products were developed using fibrinogen at 10% and thrombin at 1% of the total weight of the meat block. Ground products developed had a larger surface area and required a greater amount of fibrin.

Product Development

Diaphragm Steak-like Product

A steak-like product was made using pork diaphragms and beef fibrin. A sensory test was conducted before the product was developed to assure the most ideal processing and cookery methods were used. The side of the diaphragm that touched the ribcage contained a thick piece of connective tissue that was not removed by the plant. Connective tissue was removed to improve tenderness and to better imitate the composition of a steak. Grilling was determined to be the most ideal method of cooking.

Raw product was made after determining the ideal processing and cooking methods. A 15 cm x 30 cm x 6 cm pan was used as the mold to form a loaf with the diaphragm. A total of 2.26 kg of diaphragm meat with the connective tissue removed, 226 g (10%) of fibrinogen, and 23 g (1%) of thrombin were thoroughly mixed by hand for one minute. Pieces of diaphragm were layered in the pan quickly before the fibrinogen and thrombin began setting. Plastic wrap was placed over the top layer and a pre-cut cardboard cap was placed on the top of the plastic wrap. The pan was then placed in a vacuum sealable bag and a vacuum was drawn. Vacuum helped

eliminate air pockets between the pieces of meat which ultimately maximizes binding area. The vacuum sealed product was then placed in the cooler at 2°C for 12 hours to allow the binding process to complete.

Final product was removed from the bag and pan after12 hrs of required setting time and sliced into 2.54 cm steaks and then grilled. After cooking, steaks maintained structural integrity and fibrin held meat together effectively resulting in no holes or weak binding points within steaks. Refer to figure 4.1 for pictures of the raw and cooked final product.

Boneless Baby-Back Rib-like Product (Jowl)

A boneless baby-back rib-like product was developed using jowl trimmings from pork carcasses. Jowls that are processed in plants are skinless and appear to have a very minimal amount of fat trimmed off; therefore, jowls used to make boneless baby-back rib-like products had approximately 20% of their fat removed before being added to the final product in order to increase the lean point. Each individual piece was placed in an electric meat tenderizer. A total of 13.6 kg of tenderized jowl trimmings, 1.36 kg (10%) of fibrinogen, and 136 g (1%) of thrombin were thoroughly mixed for about one minute. Pieces were layered in a 50 cm x 30 cm x 6 cm loaf pan. Plastic wrap was placed over the top layer and a pre-cut cardboard cap was placed over the top of the plastic wrap. Final product was placed in a vacuum sealable bag and a vacuum was drawn. Product was placed in the cooler at 2°C for at 12 hrs to allow the binding process to complete.

Product was placed in the freezer at -20°C after initial 12 hrs in the cooler. The binding process must be complete before a product is frozen or weak points between the pieces of meat developed. The frozen boneless baby back loaf was then tempered until the internal and external temperatures fell within the range of -4.4°C to -2.2°C. This temperature range reduced the chance

of tearing the meat with the knife. The loaf was cut into 2.54 cm thick pieces using a custom edged knife with a zig-zag design which cut the meat to imitate the look of a traditional bone-in baby back rib. The final product was best when grilled. Refer to figure 4.2 for pictures of the raw and cooked final product.

Bung Roll (Liver, Kidney, Heart)

The bung roll was developed to test the binding capability of fibrin on offal meats that contained a high percentage of water. Whole livers, hearts, and kidneys were selected from pork carcasses and then coarse ground together at a ratio of 1:1:1. Flushed pork bungs were tied off at one end, soaked in saltwater, and used as the casing for the ground livers, hearts, and kidneys. Ground offals were weighed and fibrinogen was added at 12% of the weight of the meat block and thrombin was added at 1.2%. The mixture was thoroughly tumbled by hand for approximately one minute before being placed in the cylinder of a hand stuffer. A bung was placed over the stuffing tube and filled to a level where the bung could be tied off at the open end. The bung roll was placed in a vacuum sealable bag and a vacuum was drawn. Product was then placed in the cooler at 2°C for 12 hours to allow the binding process to complete.

The bung roll was removed from the cooler after the required 12 hrs of chilling. The roll was further processed in three different ways. The first attempt involved slicing the chilled bung roll that had an internal temperature of 2.2° C. Slicing did not work because it appeared as though there was too much purge to allow the fibrin to set appropriately. For the second processing method the bung roll was frozen at -20°C, tempered to -3.3°C, and then sliced at 0.6 cm. Slices held together and remained one solid piece after frying for approximately 10 min. The most ideal processing method was to cook the bung roll in an oven set to 149°C and 100% humidity until an internal temperature of 82°C was reached. Offal meat within this bung roll

remained intact when sliced with a knife. Refer to figure 4.3 for pictures of the raw and cooked final product.

Heart and Fat Steak

The heart and fat steak was developed to determine fibrin effectiveness with a low moisture ground product. Pork back fat and hearts that had large visible veins removed were coarse ground once and then mixed together by hand for 3 min. A total of 907 g of heart and 340 g of fat were used to fill a 15 cm x 30 cm x 6 cm loaf pan. Fibrinogen and thrombin were added at a rate of 12% and 1.2% respectively to the ground product and then mixed again thoroughly by hand for approximately 1 min. Plastic wrap was placed over the top layer and a pre-cut cardboard cap was placed on the top of the plastic wrap. Final product was then placed in a vacuum sealable bag and a vacuum was drawn. Product was then placed in the cooler at 2°C for 12 hrs to allow the binding process to complete.

Final product was removed from the cooler after 12 hrs and then removed from the bag and pan. The loaf was sliced into 2.54 cm thick steaks and the steaks were grilled. Steaks remained intact and had very few weak points between the meat and fat particles. Refer to figure 4.4 for pictures of the raw and cooked final product.

Boneless Hock

The boneless hock is an example of how a cut that is typically sold with the bone intact can be deboned and bound together to form one solid piece that consumers may find more acceptable. The boneless hock was perhaps the most practical product developed since it ultimately represents products such as bone-in hams which could potentially lead to opportunities in both foreign and domestic markets. Pork packers that sell the hocks market them as bone-in. To make the boneless hock, the bone was removed from the meat portion of the hock

and a mixture of 10% fibrinogen and 1% thrombin was rubbed into the center portion of the hock. The hock was then folded back into its original shape, placed in a vacuum package bag, and finally sealed. Final product was left in the cooler at 2°C for 12 hrs. Refer to figure 4.5 for pictures of the final product.

Sliced Tongue

A log containing skinned pork tongues was developed to show binding capabilities of whole muscles within a synthetic casing. Sliced beef tongue is a popular Asian dish and the result of binding pork tongues together is a larger diameter product that is more similar in size to a beef tongue. A total of 2.7 kg of skinned pork tongues were combined with a mixture containing 10% fibrinogen and 1% thrombin. Tongues and fibrin were tumbled by hand for approximately 1 min before being layered into an Aligned Grain Stuffer (AGS). A 7.6 cm diameter perforated synthetic casing that was tied off at one end was placed over the mouth of the AGS. The perforated casing helps remove air pockets that may result and prevents the need for vacuum sealing. The air powered AGS was turned on and stuffed the aligned tongues into the casing. The casing was tied off and the final product was left to chill for 12 hours in the cooler at 2°C.

The tongue log was removed after the appropriate chilling period and the casing was removed. The log was cut into 0.3 cm slices using a deli meat slicer and 0.6 cm slices using a knife. Slices were then cooked in a frying pan to 74°C. The slices remained intact and did not have any weak points between pieces. Refer to figure 4.6 for pictures of the raw and cooked final product.

Diaphragm Pinwheel

The diaphragm pinwheel is a novelty item that shows fibrin will bind meat, even when other ingredients are incorporated. First, skinned diaphragms and a slurry containing 10% fibrinogen and 1% thrombin were hand-mixed together for approximately one minute. Two pieces of diaphragm were used to create each pinwheel. The two pieces were lined up and overlapped each other by 5 cm. Outside ingredients added to the pinwheels include cheese, orange bell pepper slices, and cilantro. These three ingredients were added to one side of the diaphragm strips and gaps between the ingredients were left in order to allow for the protein to protein contact that is necessary for binding. Continuous diaphragm strip was then rolled up on itself to keep the added ingredients inside the pinwheel. The pinwheel was placed in a round plastic form, vacuum sealed in a bag, and then placed in the cooler at 2°C for 12 hrs to allow the binding process to complete.

The pinwheel was removed from the bag and the plastic form after the appropriate chilling period. The pinwheel was grilled to test the fibrin's ability to continue to hold together both the meat and the added ingredients. Product remained intact and maintained its original structure through the cooking process. Refer to figure 4.7 for pictures of the raw and cooked final product.

Jowl Bacon

The jowl bacon is a product derived from the same form as the boneless baby back. Although the bacon procured for this product is fresh and uncured, similar cured products such as hams have been procured using fibrin making a cured bacon-like product a possibility (Romero de Avila et al., 2014). The same procedures were used to make the bacon; however, the frozen jowl loaf was sliced using a vertical band saw at 0.3 cm. The same jowl loaf can be

thawed and sliced using a meat slicer as an alternative. Refer to figure 4.8 for pictures of the raw and cooked final product.

Results and Discussion

The purpose of this research was to determine whether or not fibrin could serve as an effective binder for pork variety and offal meats. Prior to this research, no work had been completed using fibrin with pork variety and offal meats. Fibrin was added to a wide array of different variety and offal meats to determine its efficacy on products containing different levels of water and collagen. Application directions and production advice from Boles and Shand (1999), Lennon et al. (2010), and Paardekooper and Wijngaards (1986) were utilized to define the process when making the final products.

As expected, pork fibrin served as an effective binder in development of all final pork products. Paardekooper and Wijngaards (1986) proved fibrin effectively binds whole muscles together such as pork shoulder muscles and rolled beef, which is similar in concept to the boneless hock, diaphragm steak, tongue log, and diaphragm pinwheels. Boles and Shand (1999) used fibrin to create restructured steakettes from course ground inside rounds, chuck tenders, chuck clods, and tri-tip which mimicked some of the products developed over the course of this project such as the bung roll and the heart and fat steak. In the end, a total of eight unique products were successfully developed using fibrin and the pork variety/offal meats listed in Table 4.1. Figures 4.1 - 4.8 show both the raw and cooked form of the products.

Products that were developed are prime examples of potential export items that could be made in plants across the U.S. The stipulations for this project required the use of commonly exported items which is why we chose to use only pork variety and offal meats. However, research has shown that fibrin can be successfully used to combine more traditional products

such as skeletal muscles. Limitations to fibrin seem to be relatively unknown and further research determining optimum inclusion levels, sensory evaluations on different product types, and the efficacy fibrin has with cured products could benefit the industry in the future. Potential products that utilize fibrin's binding capabilities can range anywhere from a low-sodium boneless deli ham to a formed imitation bacon product made from pork jowls. Results from this study suggest that fibrin is a feasible naturally-occurring binder that could be utilized by the U.S. pork industry to create value-added products.

Table 4.1. List of products utilized as common pork export items.

Jowls	Hocks	Skins
Kidneys	Tongues	Livers
Diaphragm (skirt) meat	Jowls	Fat-Back
Hearts	Rectum	

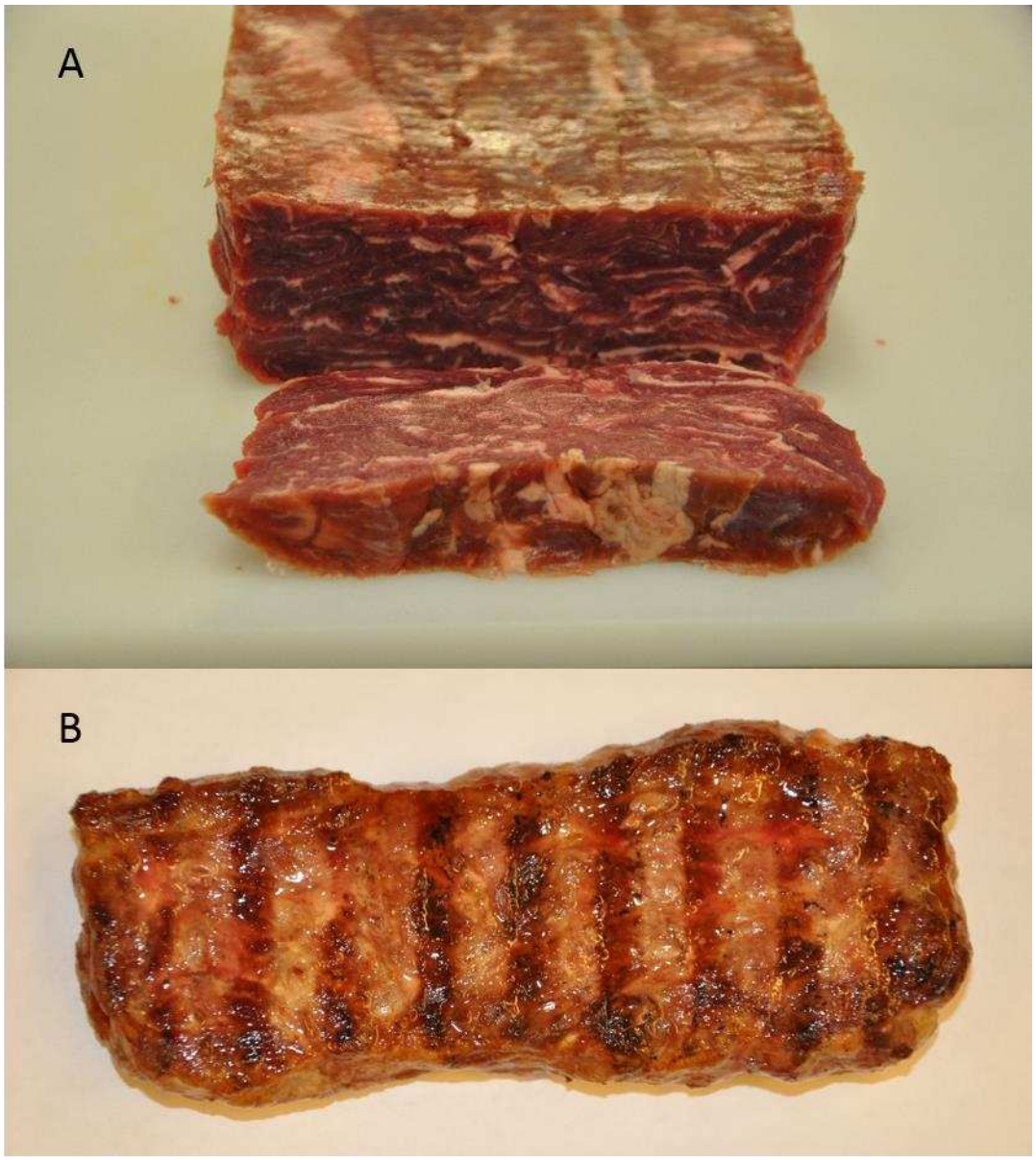

Figure 4.1. Diaphragm Steak. Raw (A) Cooked (B)

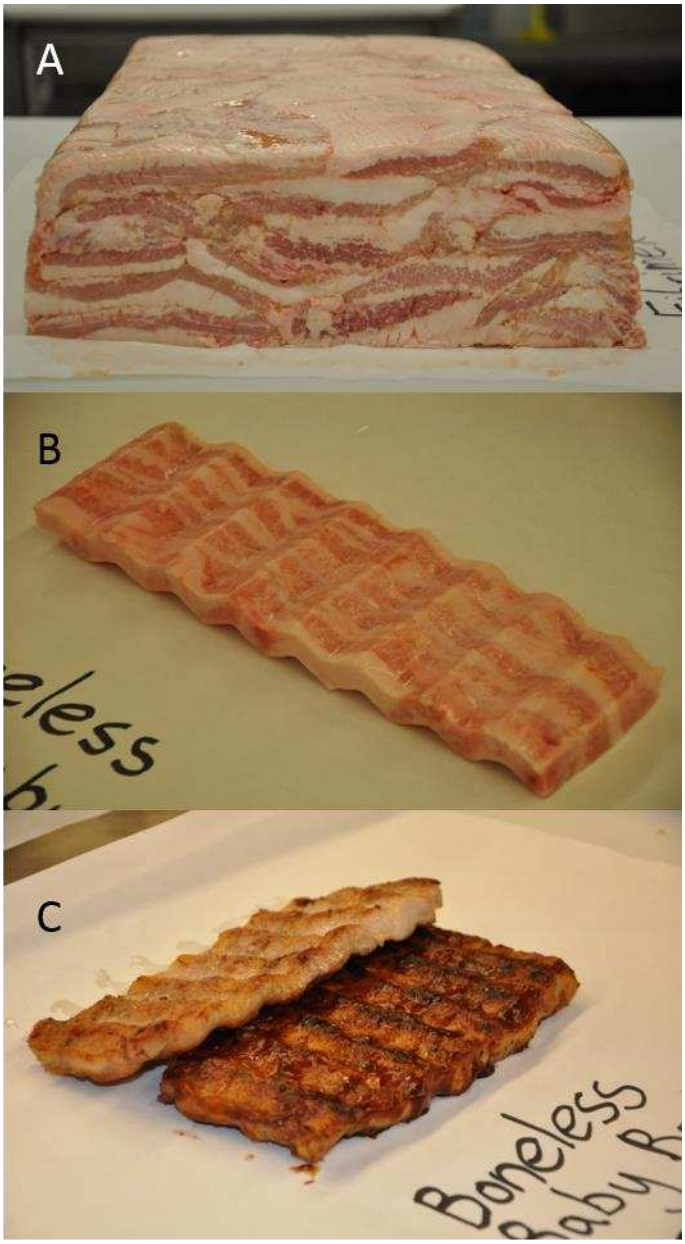

Figure 4.2. Boneless Baby Back. Raw Loaf (A) Raw (B) Cooked (C)

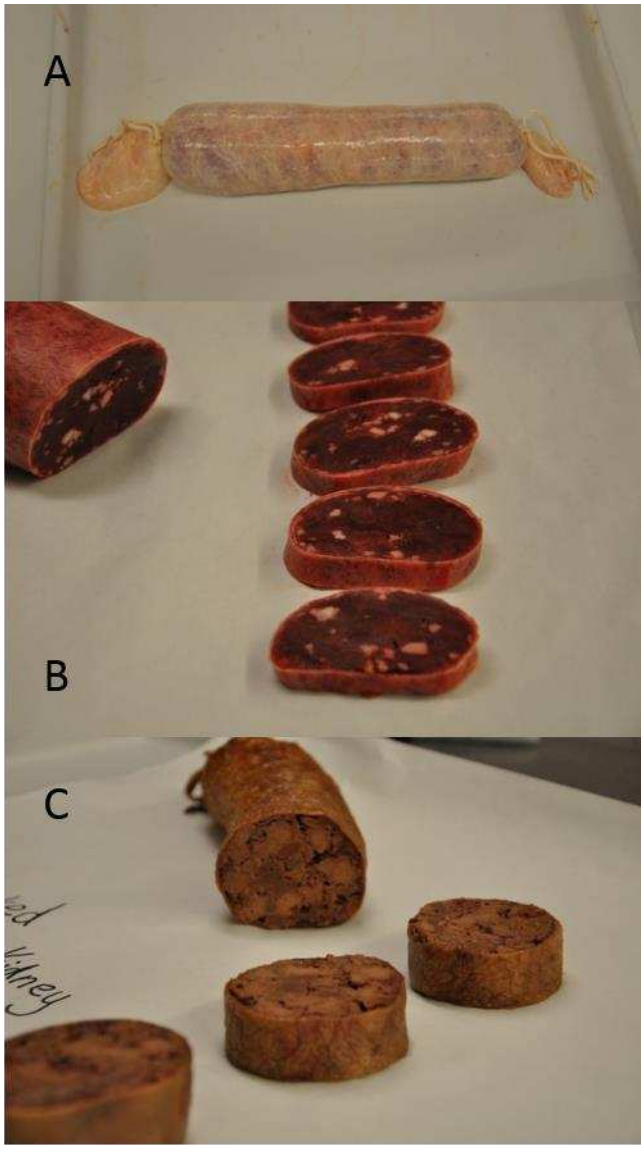

Figure 4.3. Bung Roll. Raw (A) Raw Slices (B) Cooked (C)

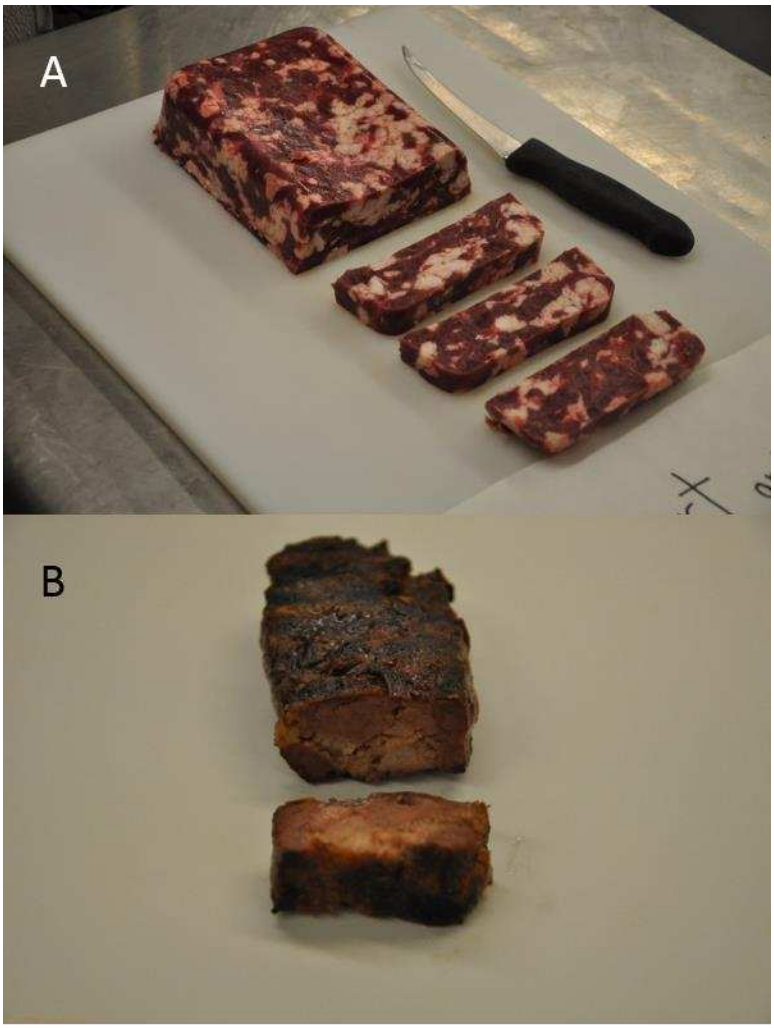

Figure 4.4. Heart and fat steak. Raw (A) Cooked (B)

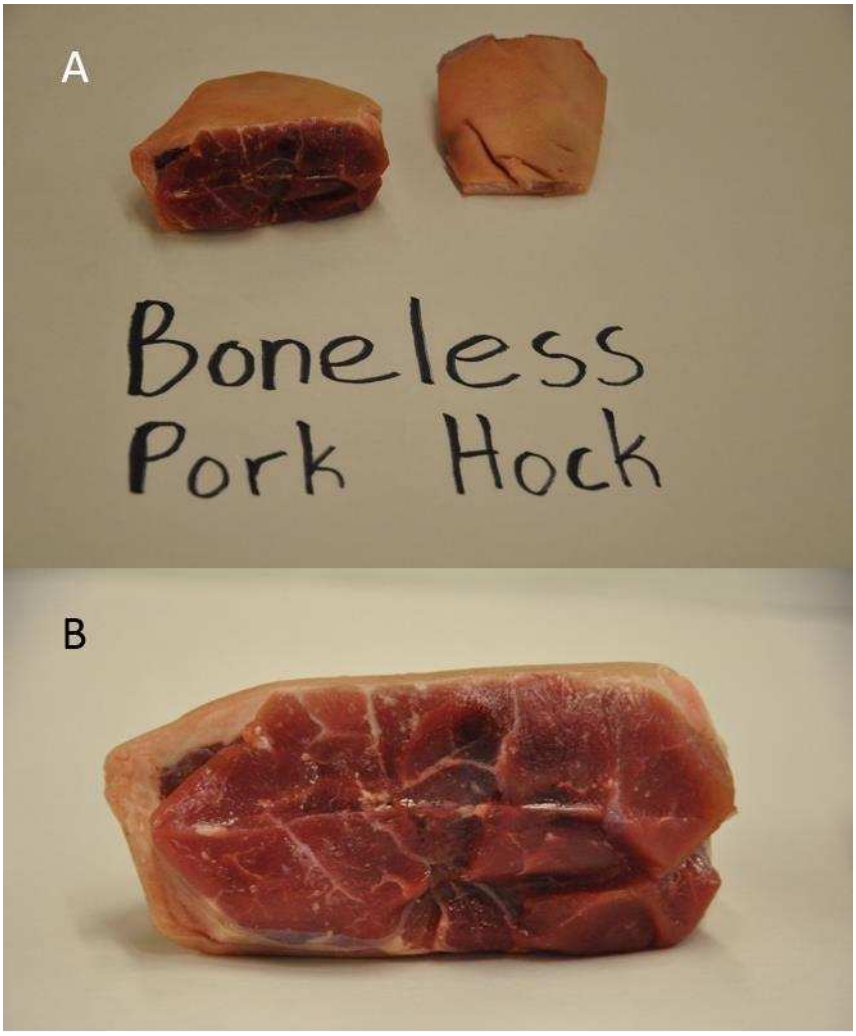

Figure 4.5. Boneless Pork Hock. Raw (A and B)

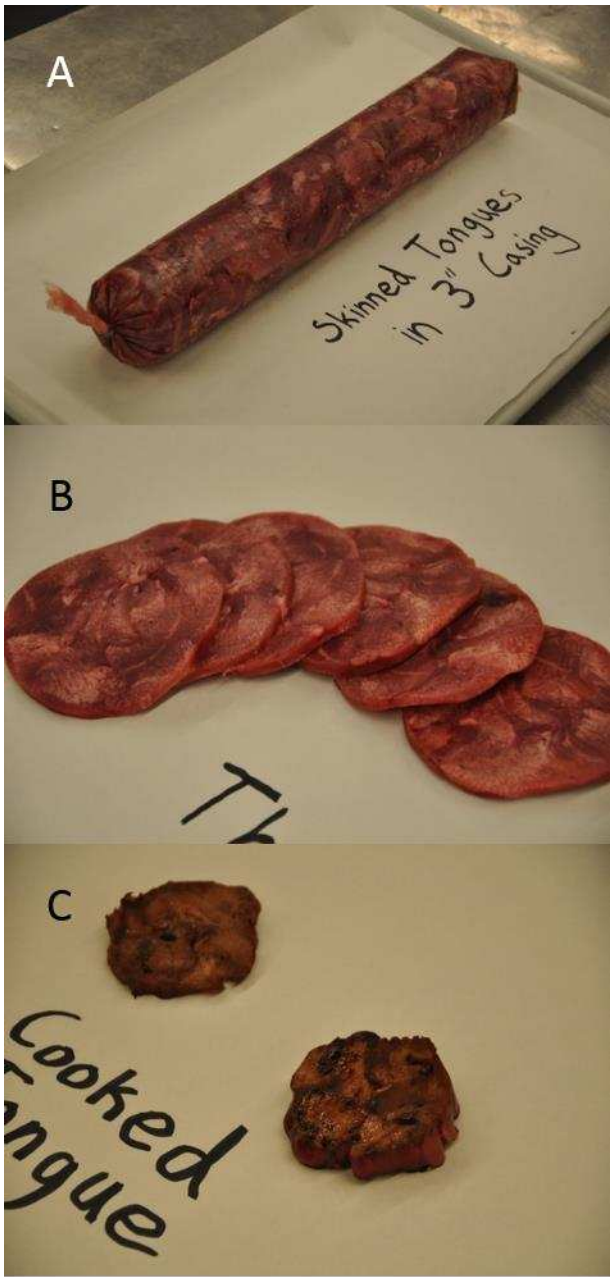

Figure 4.6. Sliced Tongue. Raw (A) Raw Slices (B) Cooked (C)

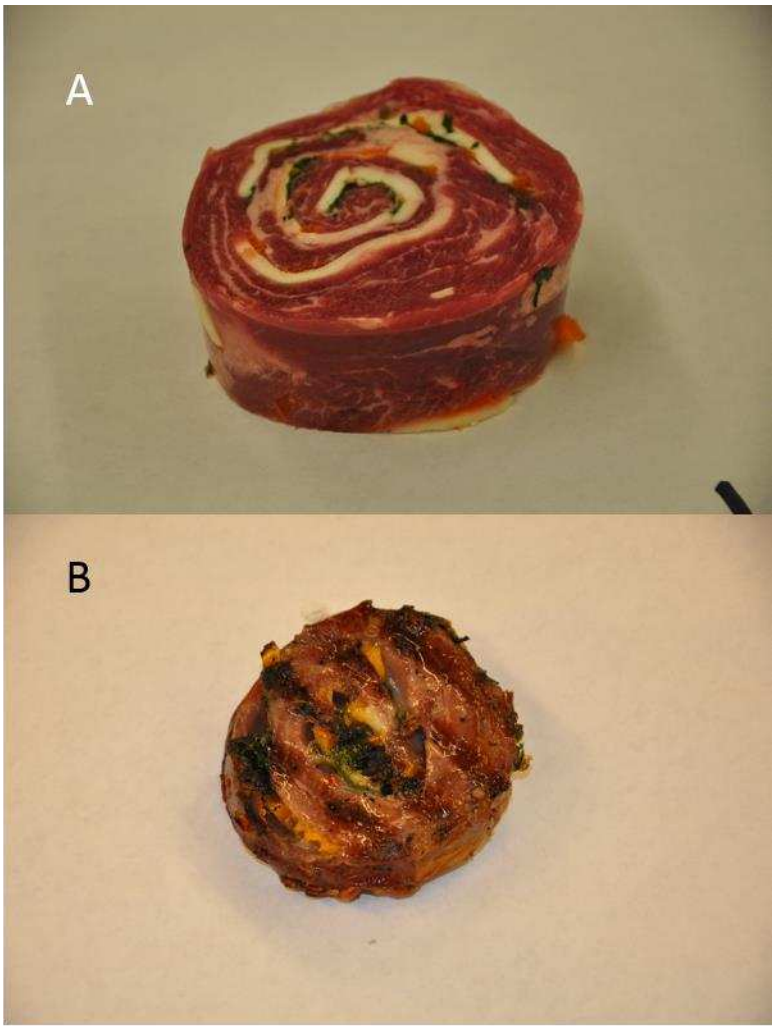

Figure 4.7. Diaphragm Pinwheel. Raw (A) Cooked (B)

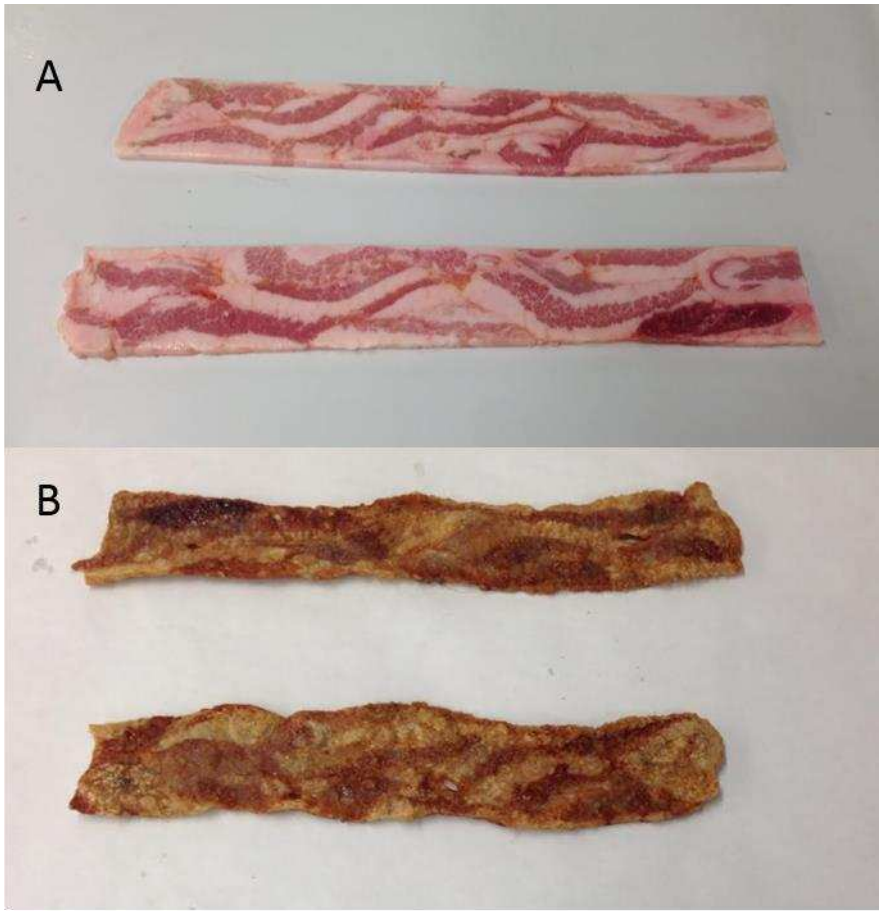

Figure 4.8. Jowl Bacon. Raw (A) Cooked (B)

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APPENDIX A

Mycobacteria Procedure

I. If samples cannot be processed immediately, place them in a -70ºC freezer or refrigerator for a maximum of 3 days.
II. If you have a **fecal** sample weigh out approximately 2 grams (walnut size) and place the sample into a 50 ml conical tube containing 35 ml of sterile water.
III. If you have a **tissue** sample mince up approximately 2 grams and place the sample into a 50 ml conical tube containing 35 ml of sterile water.
IV. Shake the conical tube vigorously and vortex for 30 minutes to allow the sample to break up.
V. After mixing, allow the tube to stand at room temperature for 30 minutes allowing the sample to settle to the bottom.
VI. Transfer 5 ml of the sample from the upper third of the tube to a second 50 ml conical tube containing 25 ml of room temperature BHI/HPC (0.75% HPC). You can discard the water tube at this point but if so desired you may save the tube under the hood in room E117 for further use.
VII. Incubate BHI/HPC tubes at 35-37ºC overnight.
VIII. After 18-24 hours centrifuge the tubes for 30 minutes at 900 x g.
IX. Pour off the supernatant and resuspend the pellet with 1 ml of the antibiotic brew and vortex well.
X. Incubate at 35-37ºC overnight (although the sample will be stable for up to 3 days)
XI. Inoculate four tubes of room temperature Herrold's Egg Yolk agar (3 with mycobactin and 1 without) with .250µl of the resuspension. Make sure you are using a different sterile pipette for each animal to avoid contamination. Roll the tubes so that the entire surface is covered with the inoculum.
XII. Incubate Herrold's Egg Yolk agar at 37ºC in a slanted position with caps loose.
XIII. After the tube has been incubated for 1-2 weeks tighten the cap and place in the upright position in the incubator.
XIV. Every 2 weeks for 16 weeks read and evaluate tube for growth/contamination. Record colony counts and describe contamination.
XV. Slightly raised white-yellow colonies appearing at 6-12 weeks should be examined with an acid fast stain to determine its acid fastness and morphological appearance.

APPENDIX B

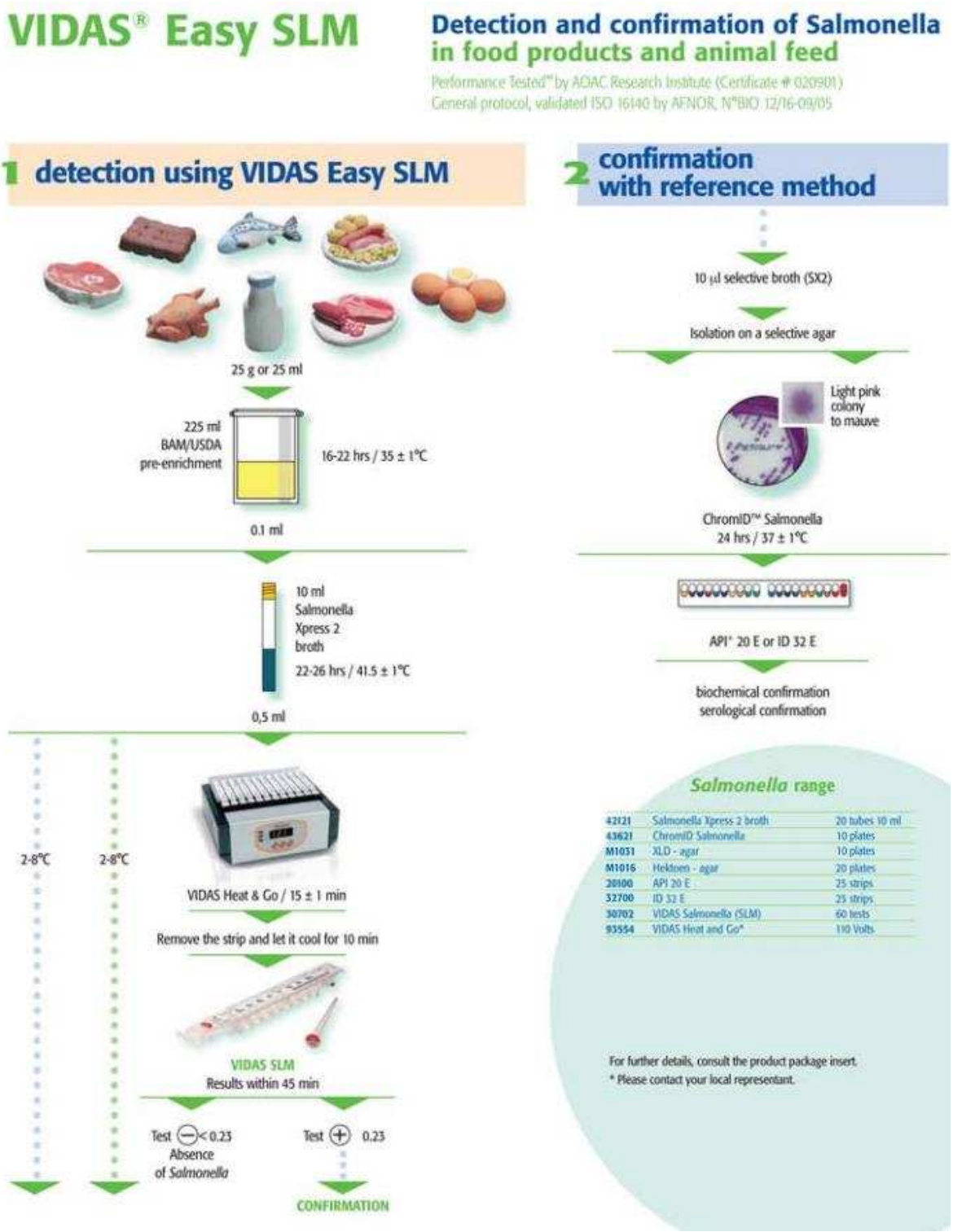
APC Procedure

The Petrifilm Aerobic Count (AC) plate is a ready-made culture medium system that contains Standard Methods nutrients, a cold-water-soluble gelling agent, and an indicator that facilitates colony enumeration. Petrifilm AC plates are used for the enumeration of aerobic bacteria.

3M Petrifilm™ Aerobic Count Plates Reminders for Use: Petrifilm Storage Sample Preparation Inoculation. Release top film; allow it to drop. Do not roll top film down. For detailed CAUTIONS, DISCLAIMER OF WARRANTIES / LIMITED REMEDY, LIMITATION OF 3M LIABILITY, STORAGE AND DISPOSAL information, and INSTRUCTIONS FOR USE see Product's package insert. Add appropriate quantity of one of the following sterile diluents: Butterfield's phosphate buffer (IDF phosphate buffer, 0.0425 g/L of KH2PO4 adjusted to pH 7.2), 0.1% peptone water, peptone salt diluent (ISO method 6887), buffered peptone water (ISO method 6579), saline solution (0.85 - 0.90%), bisulfatefree letheen broth, or distilled water. Do not use buffers containing citrate, bisulfite, or thiosulfate; they can inhibit growth. Adjust pH of the diluted sample between 6.6 and 7.2. For acid products, use 1N NaOH, for alkaline products, use 1N HCI.

APPENDIX C

***Salmonella* Procedure**



APPENDIX D

Yeast and Mold Procedures

Enumeration of Yeasts and Molds in Food--Dilution Plating Technique

A. **Equipment and materials**

1. Basic equipment (and appropriate techniques) for preparation of sample homogenate, see Chapter 1
2. Equipment for plating samples, see Chapter 3
3. Incubator, 25°C
4. Arnold steam chest
5. pH meter
6. Water bath, 45 ± 1° C

B. **Media and reagents**

Media

1. Dichloran rose bengal chloramphenicol (DRBC) agar (M183)
2. Dichloran 18% glycerol (DG18) agar (M184)
3. Plate count agar (PCA), standard methods (M124); add 100 mg chloramphenicol/liter when this medium is used for yeast and mold enumeration. This medium is not efficient when "spreader" molds are present.
4. Malt agar (MA)(M185)
5. Malt extract agar (Yeasts and Molds) (MEAYM)(M182)
6. Potato dextrose agar (PDA), dehydrated; commercially available (M127)

Antibiotic solutions

Antibiotics are added to mycological media to inhibit bacterial growth. Chloramphenicol is the antibiotic of choice, because it is stable under autoclave conditions. Therefore, media preparation is easier and faster due to the elimination of the filtration step. The recommended concentration of this antibiotic is 100 mg/liter medium. If bacterial overgrowth is apparent, prepare media by adding 50 mg/liter chloramphenicol before autoclaving and 50 mg/liter filter-sterilized chlortetracycline when the media have been tempered, right before pouring plates.

Prepare stock solution by dissolving 0.1 g chloramphenicol in 40 ml distilled water; add this solution to 960 ml medium mixture before autoclaving. When both chloramphenicol and chlortetracycline are used, add 20 ml of the above chloramphenicol stock solution to 970 ml medium before autoclaving. Then, prepare chlortetracycline stock solution by dissolving 0.5 g antibiotic in 100 ml distilled water and filter sterilize. Use 10 ml of this solution for each 990 ml of autoclaved and tempered medium. Refrigerate in the dark and re-use remaining

stock solutions for up to a month. Stock solutions should be brought to room temperature before adding to tempered medium.

C. **Procedures**

Sample preparation

Analyze 25-50 g from each subsample; generally, larger sample sizes increase reproducibility and lower variance compared with small samples. Test individual subsamples or composite according to respective Compliance Program for the food under analysis. Add appropriate amount of 0.1% peptone water to the weighed sample to achieve 10^{-1} dilution, then homogenize in a stomacher for 2 min. Alternatively, blending for 30-60 sec can be used but is less effective. Make appropriate 1:10 (1+9) dilutions in 0.1% peptone water. Dilutions of 10^{-6} should suffice.

Plating and incubation of sample

Spread-plate method. Aseptically pipet 0.1 ml of each dilution on pre- poured, solidified DRBC agar plates and spread inoculum with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

Pour-plate method. Use sterile cotton-plugged pipet to place 1.0 ml portions of sample dilution into prelabeled 15 x 100 mm Petri plates (plastic or glass), and immediately add 20-25 ml tempered DG18 agar. Mix contents by gently swirling plates clockwise, then counterclockwise, taking care to avoid spillage on dish lid. After adding sample dilution, add agar within 1-2 min; otherwise, dilution may begin to adhere to dish bottom (especially if sample is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate.

From preparation of first sample dilution to pouring or surface-plating of final plate, no more than 20 min (preferably 10 min) should elapse. **Note:** Spread plating of diluted sample is considered better than the pour plate method. When the pour plate technique is used, fungal colonies on the surface grow faster and often obscure those underneath the surface, resulting in less accurate enumeration. Surface plating gives a more uniform growth and makes colony isolation easier. DRBC agar should be used for spread plates only.

Incubate plates in the dark at 25°C. Do not stack plates higher than 3 and do not invert. **Note:** Let plates remain undisturbed until counting.

Counting of plates

Count plates after 5 days of incubation. If there is no growth at 5 days, re-incubate for another 48 h. Do not count colonies before the end of the incubation period because handling of plates could result in secondary growth from dislodged spores, making final counts invalid. Count plates containing 10-150 colonies. If mainly yeasts are present, plates with 150 colonies are usually countable. However, if substantial amounts of mold are present, depending on the type of mold, the upper countable limit may have to be lowered at the discretion of the analyst. Report results in colony forming units (CFU)/g or CFU/ml based on average count of triplicate set. Round off counts to two significant figures. If third digit is 6 or above, round off to digit above (e.g., 456 = 460); if 4 or below, round off to

digit below (e.g., 454 = 450). If third digit is 5, round off to digit below if first 2 digits are an even number (e.g., 445 = 440); round off to digit above if first 2 digits are an odd number (e.g., 455 = 460). When plates from all dilutions have no colonies, report mold and yeast counts (MYC) as less than 1 times the lowest dilution used. Isolate individual colonies on PDA or MA, if further analysis and species identification is necessary.

APPENDIX E

***Campylobacter* spp. Procedure**

BAX® System Protocol

1. Create rack file and warm up cycler.
2. Mix protease with lysis buffer and transfer 200 µL of lysis reagent to cluster tubes.
3. Transfer 5-µL samples to cluster tubes.
4. Heat cluster tubes for 20 minutes at 37°C, then 10 minutes at 95°C.
5. Cool cluster tubes for 5 minutes in cooling block, then transfer 30 uL to PCR tubes in cooling block.
6. Place sealed PCR tubes in cycler and run program.
7. Review results.

APPENDIX F

***Streptococcus suis* Procedure**

UNIVERSITY OF MINNESOTA VETERINARY DIAGNOSTIC LABORATORY Standard Operating Procedure (SOP) Doc. No.: MB.SOP.0040 Revision: 4 Category: Molecular Bacteriology Section, Test Method Active Date: 04/04/2013 Title: S. suis Detection PCR Page 1 of 5 SYS.FORM.016, REV03, 06/03/2009

1. Purpose: To outline the protocol for a PCR test to detect Streptococcus suis in clinical tissues and bacterial isolates using Qiagen Hot StarTaq Master Mix kit.

2. Responsibility: It is the responsibility of the VDL Section Head to ensure training for staff that will perform this SOP. It is the responsibility of laboratory personnel using this procedure to read, understand, receive training for, and agree to follow the procedure described in this SOP.

3. Definitions: PCR: Polymerase Chain Reaction

4. Equipment and Material: 0.2 ml MicroAmp PCR Reaction tubes PCR reaction tube holder Pipettes Pipette tips 0.65 ml microfuge tubes GeneAmp PCR System 9700 Thermalcycler or equivalent Vortex Hot StarTaq Master Mix Kit (Qiagen, Cat # 203443, or equivalent) RNase, DNase- free PCR water Primers JP4: 5' – GCA GCG TAT TCT GTC AAA CG – 3' JP5: 5' – CCA TGG ACA GAT AAA GAT GG – 3' Sample DNA Positive (+) control: S. suis (reference strain) Organism Accession # Streptococcus suis S. suis Serotype 1 1% Agarose Gel with 14 or 16 well combs (Protocol MB.SOP.0001). 1 X TAE Buffer 0.625 mg/ml Ethidium Bromide Solution 5X Loading Dye Hi-Low DNA Marker

5. Safety: 5.1 Training for this procedure includes review of hazards and accident prevention, personal protective equipment (PPE) and other safety requirements based on a risk assessment of the specific methods, reagents, and/or equipment used. Specific requirements may be found in the body of the document. 5.1.1 Personal Protective Equipment (PPE) includes: 5.1.1.1 Laboratory coat 5.1.1.2 Non-Latex, powder free gloves 5.2 University of Minnesota safety information and safety policies are available from the U of M Department of Environmental Health and Safety (DEHS) on their website (www.dehs.umn.edu). 5.3 Material Safety Data Sheets (MSDS) are available in a labeled binder in VDL Molecular Bacteriology Room 340. 5.3.1 Hazardous chemicals used in this Procedure include: 5.3.1.1 Agarose (irritant to eyes/skin/respiratory tract) 5.3.1.2 Ethidium bromide solution (irritant to skin/eyes/respiratory system, toxic to ingest, potent mutagen) 5.3.1.3 TAE Buffer 50X (irritant to skin/eyes/respiratory tract) 5.3.1.4 Loading Dye (irritant to skin, eyes and respiratory tract, may affect kidneys) 5.4 All biological, chemical, and radioactive waste is disposed according to state, federal, and U of M requirements as found at www.dehs.umn.edu "Hazardous Waste".

6. Training: Laboratory personnel will receive training and will follow appropriate document review schedule. Training status is maintained within the sections and the SOP revision records are archived in the VDL Q-Pulse Document module.

7. Procedure: 7.1 Please refer to MB.SOP.0065 Cleaning Procedures, and/or MOL.SOP.306 Contamination Prevention for the proper method of sample/reagent handling and proper directional flow. 7.2 Obtain extracted DNA to be run (see SOP protocol MB.SOP.00013, Microbial DNA Extraction from Tissue or MB.SOP.0032 Microbial DNA Extraction from Gram

Positive Pure Cultures). 7.3 Record lot numbers and pipette numbers on the S. suis PCR Test Sheet (see MB.FORM.0018). 7.4 Record PCR tube number, along with the DNA extraction number, the VDL accession number, and any notes on the S. suis PCR Test Sheet. 7.5 Prepare primer stock if necessary (see protocol MB.SOP.0028 Standard Primer Dilution). 7.6 Calculate the volumes of master mix components and record in the table: Master Mix, using the following guidelines: Component Working Concentration Volume per 25 µl Reaction (µl) Final Concentration Hot StarTaq mixture * Note 1 12 *Note 1 JP4 F primer 10 µM 0.8 0.32 µM /µl JP5 R primer 10 µM 0.8 0.32 µM /µl PCR Water N/A 9.4 N/A Final Volume N/A 23 N/A DNA N/A 2 *Note 2 N/A *Note 1: For HotStarTaq components and concentrations see: Hot StarTaq PCR Handbook (October 2005) *Note 2: Add 2 µl of extracted DNA, no need to quantify. 7.7 Prepare PCR master mix by vortexing each constituent and pipetting calculated volume into microfuge tube. 7.8 Vortex mixture well. 7.9 Clearly label 0.2 ml PCR reaction tubes with the PCR tube number and place them in a PCR reaction tube holder and tray. 7.10 Aliquot 23 µl of prepared master mix into each PCR tube. 7.11 Add 2 µl of sample template DNA into the respective PCR tube and mix well. Make sure to add the S. suis positive control, as well as a negative control, for each PCR run. 7.12 Remove the PCR reaction tubes and holder from the tray and place into the GeneAmp PCR System 9700 Thermalcycler. 7.13 Run program: s. suis detection pcr under username: simone: using reaction volume of 25 µl. (See MB.SOP.0006 Operation instructions for the GeneAmp PCR 9700 Thermalcycler). S. suis Detection PCR Program: 1 st Hold: 35 cycles of: 2nd Hold: 95 C for 15 min 94 C for 30sec 72 C for 10 min 55 C for 1 min 72 C for 2 min 7.14 Run Gel Electrophoresis for detection PCR: Use 12 l PCR product from each sample for electrophoresis in a 1% TAE-agarose gel stained with ethidium bromide. 7.14.1 Prepare a 1% gel as outlined in MB.SOP.0001 (Agarose Gel Prep with Ethidium Bromide Solution) depending on size requirements and the number of samples. 7.14.2 Record the lane number, and the well number, with the respective PCR tube number, the DNA extraction tube number and the VDL accession number in the Gel ID Map table (MB.FORM.0003). 7.14.3 When the gel has solidified and casting ends have been removed, place gel in gel box with enough fresh 1 X TAE buffer to cover the wells. 7.14.4 Mix 2 µl loading dye into each PCR tube. 7.14.5 Remove the gel comb(s) 7.14.6 Add 10 µl High-Low DNA Marker into the first well of each lane used. 7.14.7 Mix PCR product and dye by pipetting up and down 3 times, and add 12 µl into each respective well following the gel ID map table. 7.14.8 Put cover on gel box and turn on power supply. 7.14.9 Run gel until leading dye reaches at least 2.0 cm (around 30 minutes). 7.15 Capture gel image digitally and on film (MB.SOP.0005, Gel Image Capturing)

8. Acceptance Criteria: 8.1 Any abnormalities or departures from normal or specified conditions as described in this test method shall be recorded. 8.2 If a positive control for a PCR test yields a negative result, then the PCR test must be repeated. Likewise, if a negative control yields a positive result, then the PCR test must be repeated. 8.3 If a positive control begins to deteriorate (produces weak band) due to degradation of DNA, then a new positive control will be will be extracted, tested and used for PCR reaction.

9. Interpretation of Results: 9.1 A sample is considered positive based on the presence of a band at approximately 688 base pairs. A sample is considered negative based on the absence of a band at approximately 688 base pairs. 9.2 Results are reported into the LIMS (see MB.REF.0001

APPENDIX G

Yersenia spp. Procedure

A.

Equipment and materials

1. Incubators, maintained at 10 ± 1°C, ± 35-37°C
2. Blender, Waring or equivalent, 8000 rpm, with 500 ml-1 liter jar
3. Sterile petri dishes, 15 x 100 mm
4. Microscope, light 900X and illuminator
5. Disposable borosilicate tubes, 10 x 75mm; 13 x 100mm.
6. Wire racks to acommodate 13x100mm tubes.
7. Vortex mixer.

B. Media

1. Peptone sorbitol bile broth (PSBB) (M120)
2. MacConkey agar (M91) (use mixed bile salts; BBL Mac agar and DIFCO Mac CS are acceptable)
3. Celfsulodin-irgasan-novobiocin (CIN) agar (M35)
4. Bromcresol purple broth (M26) supplemented individually with the following carbohydrates, each at 0.5%: mannitol, sorbitol, cellobiose, adonitol, inositol, sucrose, rhamnose, raffinose, melibiose, salicin, xylose, and trehalose
5. Christensen's urea agar (M40) (plated media or slants)
6. Phenylalanine deaminase agar (M123) (plated media or slants)
7. Motility test medium (M103). Add 5 ml of 1% 2,3,5-triphenyl tetrazolium chloride per liter before autoclaving.
8. Tryptone broth, 1% (M164)
9. MR-VP broth (M104)
10. Simmons citrate agar (M138)
11. Veal infusion broth (M173)
12. Bile esculin agar (M18)
13. Anaerobic egg yolk agar (M12)
14. API 20E or Vitek GNI
15. Trypticase (tryptic) soy agar with yeast extract (TSAYE) (M153)
16. Lysine arginine iron agar (LAIA) (M86)
17. Decarboxylase basal medium (Falkow) (M44) supplemented with 0.5% ornithine
18. Congo Red-brain heart infusion agarose (CRBHO) (M41)

19. Pyrazinamidase agar slants (M131)
20. PMP broth (M125)
21. β-D-glucosidase test (**see** instructions at end of chapter)

C. Reagents

1. Gram stain reagents (R32)
2. Voges-Proskauer (VP) test reagents (R89)
3. Ferric chloride, 10% in distilled water (R25)
4. Oxidase test reagent (R54)
5. Saline, 0.5% (sterile) (R66)
6. Kovacs' reagent (R38)
7. 0.5% Potassium hydroxide in 0.5% NaCl, freshly prepared
8. Mineral oil, heavy grade, sterile (R46)
9. API 20E system or Vitek system with GNI cards (bioMerieux)
10. 1% Ferrous ammonium sulfate

D. Enrichment

The following simplified procedure for isolating *Yersinia* from food, water, and environmental samples is recommended.

1. Analyze samples promptly after receipt, or refrigerate at 4°C. (Freezing of samples before analysis is not recommended, although *Yersinia* have been recovered from frozen products.) Aseptically weigh 25 g sample into 225 ml PSBB. Homogenize 30 s and incubate at 10°C for 10 days.
2. If high levels of *Yersinia* are suspected in product, spread-plate 0.1 ml on MacConkey agar (15,55) and 0.1 ml on CIN agar (47,54) before incubating broth. Also transfer 1 ml homogenate to 9 ml 0.5% KOH in 0.5% saline (4), mix for 2-3 seconds, and spread-plate 0.1 ml on MacConkey and CIN agars. Incubate agar plates at 30°C for 1-2 days.
3. On day 10, remove enrichment broth from incubator and mix well. Transfer one loop-full of enrichment to 0.1 ml 0.5% KOH in 0.5% saline and mix for 2-3 s (4). Successively streak one loopful to MacConkey plate and one loopful to CIN plate. Transfer additional 0.1 ml enrichment to 1 ml 0.5% saline and mix 5-10 s before streaking, as above. Incubate agar plates at 30°C for 1-2 days.

E. Isolation of *Yersinia*

Examine MacConkey agar plates after 1 to 2 days incubation. Reject red or mucoid colonies. Select small (1-2 mm diameter) flat, colorless, or pale pink colonies.

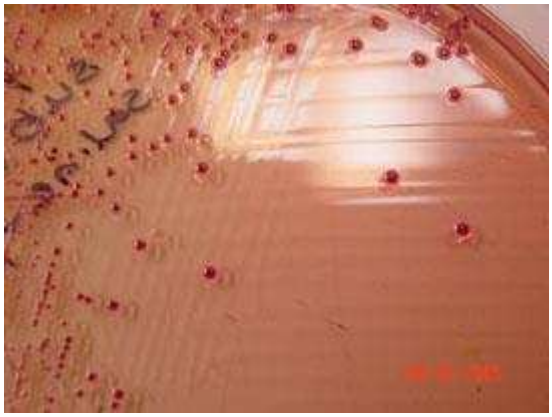
***Y. enterocolitica* on MacConkey agar**

- **Lactose negative colonies**
- **flat, colorless, or pale pink**
- **1-2 mm diameter**

Examine CIN plates after 1 day incubation. Select small (1-2 mm diameter) colonies having deep red center with sharp border surrounded by clear colorless zone with entire edge.

***Y. enterocolitica* colonies on YSA (CIN) agar**

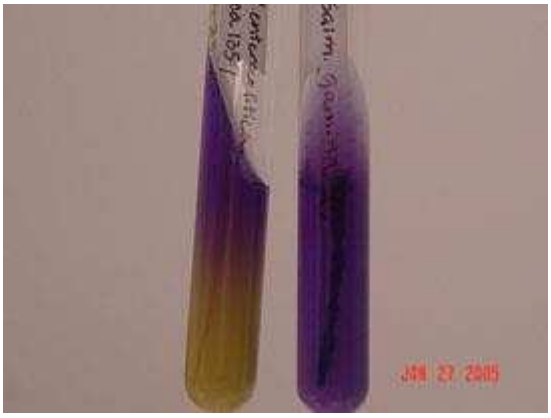
- **deep red center**
- **Surrounded by clear, colorless zone**
- **1-2 mm diameter**



Inoculate each selected colony into LAIA slant (53), Christensen's urea agar plate or slant, and bile esculin agar plate or slant by stabbing with inoculation needle. Incubate 48 h at RT. Isolates giving alkaline slant and acid butt, no gas and no H_2S (KA− −) reaction in LAIA, which are also urease-positive, are presumptive *Yersinia*. Discard cultures that produce H_2S and/or any gas in LAIA or are urease-negative. Give preference to typical isolates that fail to hydrolyze (blacken) esculin.

LAIA Slant

- ***Y. enterocolitica*** **(left) = K A − −**
- ***Salmonella*** **(right) = K K + −**



Christensen's Urea agar

- ***Y. ent.*** **= pink color (urease postitive)**
- ***E. coli*** **= no color (urease negative)**

Bile Esculin agar

- ***Y. ent.*** **(except biotype 1A) are esculin negative(absence of black color)**
- ***Ent. faecalis*** **= esculin positive (black color)**

F. Identification

Using growth from LAIA slant, streak culture to one plate of TSAYE and incubate at RT. Use growth on AEY to check culture purity, lipase reaction (at 2-5 days), oxidase test, Gram stain, and inoculum for biochemical tests. From colonies on TSAYE, inoculate the following biochemical test media and incubate all at RT for 3 days (except one motility test medium and one MR-VP broth, which are incubated at 35-37°C for 24 h).

1. Decarboxylase basal medium (Falkow) (M44), supplemented with each of 0.5% lysine, arginine, or ornithine; overlay with sterile mineral oil
2. Phenylalanine deaminase agar (M123)
3. Motility test medium (semisolid) (M103), 22-26°C and 35-37°C

Motility Test Medium with TTC

- ***Y. ent.* are motile at 25°C (2 left tubes) and non-motile at 35°C (2 right tubes)**

4. Tryptone broth (M164)
5. Indole test (**see** instructions at end of chapter)
6. MR-VP broth (M104). RT for autoagglutination test (**see** H1, below), followed by V-P test (48 h) (**see** instructions at end of chapter); 35-37°C for autoagglutination test (**see** H-1)
7. Bromcresol purple broth (M26) with 0.5% of the following filter-sterilized carbohydrates: mannitol, sorbitol, cellobiose, adonitol, inositol, sucrose, rhamnose, raffinose, melibiose, salicin, trehalose, and xylose
8. Simmons citrate agar (M138)
9. Veal infusion broth (M173)
10. Use API 20E system or Vitek GNI for biochemical identification of *Yersinia*. Follow manufacturer's instructions. These systems are generally reliable to identify *Yersinia* to genus level but are generally unreliable in identification of*Yersinia* to species level (3, 32). Use conventional biochemical tests for speciation and biotyping of presumptive virulent isolates. Biochemical tests that are important for speciation within the genus *Yersinia* are fermentation of sucrose, rhamnose, raffinose and melibiose and the utilization of citrate (Table 1). Biochemical tests important for biotyping are

fermentation of salicin, xylose and trehalose along with VP reaction, lipase, esculinase, β-D-Glucosidase, and pyrazinamidase (Table 2).

11. Pyrazinamidase agar slants (48 h) (**see** instructions at end of chapter)
12. β-D-glucosidase test (30°C, 24 h) (**see** instructions at end of chapter)
13. Lipase test. When grown on agar media containing egg yolk such as Anaerobic egg yolk agar, colonies may exhibit lipase activity. A positive reaction is indicated by oily, iridescent, pearl-like colony surrounded by precipitation ring and outer clearing zone.

B. Interpretation

Yersinia are oxidase-negative, Gram-negative rods. Use Tables 1 and 2 to identify species and biotype of *Yersinia*isolates. Currently only strains of *Y. enterocolitica* biotypes 1B, 2, 3, 4, and 5 are known to be pathogenic. These biotypes and *Y. enterocolitica* biotype 6 and *Y. kristensenii* do not rapidly (within 24 h) hydrolyze esculin or ferment **salicin**(Tables 1 and 2). However, *Y. enterocolitica* biotype 6 and *Y. kristensenii* are relatively rare; they can be distinguished by failure to ferment sucrose, and they are pyrazinamidase-positive (28). Hold *Y. enterocolitica* isolates which are within biotypes 1B, 2, 3, 4, and 5 for further pathogenicity tests.

C. Pathogenicity testing

1. **Autoagglutination test**. The MR-VP tube incubated at RT for 24 h should show some turbidity from bacterial growth. The 35-37°C MR-VP should show agglutination (clumping) of bacteria along walls and/or bottom of tube with clear supernatant fluid. Isolates giving this result are presumptive positive for the virulence plasmid. Any other pattern for autoagglutination at these two temperatures is considered negative.

MRVP Agglutination Test

- **When grown in MRVP broth at 25° C, pathogenic *Y. ent.*displays diffuse growth (left tube) but at 35°C cells agglutinate and settles to the bottom (right tube)**

2. **Freezing cultures**. Plasmids that determine traits related to pathogenicity of *Yersinia* can be spontaneously lost during culture above 30°C or with lengthy culture and passage below 30°C in the laboratory. It is important, therefore, to immediately freeze presumptive positive cultures to protect plasmid content. Inoculate into veal infusion broth and incubate 48 h at RT. Add 10% sterile glycerol (e.g., 0.3 ml in 3 ml veal infusion broth) and freeze immediately. Storage at -70°C is recommended.

3. **Low calcium response Congo Red agarose virulence test**. Inoculate test organism into BHI broth. Incubate overnight at 25-27°C. Make decimal dilutions in physiologic saline to obtain 1,000 cells/ml. Spread-plate 0.1 ml of appropriate dilution on each of two Congo Red agarose plates. Incubate one at 35°C and one at 25°C. Examine at 24 and 48 h. Presumptive plasmid-bearing *Y. enterocolitica* will appear as pinpoint, round, convex, red, opaque colonies. Plasmidless *Y. enterocolitica* will appear as large, irregular, flat, translucent colonies.

***Y. enterocolitica* on CRBHO After 24h at 35° C**

- **Plasmid bearing *Y. ent.* colonies are pinpoint convex, red, opaque.**
- **Plasmidless colonies are large, irregular, flat, and translucent.**

4. **Intraperitoneal infection of adult mice pretreated with iron dextran and desferrioxamine B.** A positive result from any of the *in vitro* pathogenicity tests (H, 1-3 above) is strong evidence of pathogenicity. These results may be confirmed by a biological test using the i.p. infection of adult mice which have been pretreated with iron dextran and the iron sequestering siderophore, desferrioxamine B. This test is described elsewhere in detail (13, 53) and is omitted here for brevity and because few labs have the facilities to perform bioassays.
5. **Invasiveness**. An in vitro HeLa cell assay is available for screening *Yersinia* isolates for invasive potential (33, 34). Acridine orange is used to stain infected HeLa cell monolayers, which are then examined under fluorescence microscope for the presence of intracellular *Yersinia* (33, 34). This in vitro staining technique can be used to determine invasiveness in both *Y. enterocolitica* and *Y. pseudotuberculosis* (16).

D. Interpretation

A positive reaction for any of the pathogenicity tests in H, 1-4 above can be taken as evidence of potential pathogenicity for a *Y. enterocolitica* or *Y. pseudotuberculosis* isolated strain.

E. *Yersinia pseudotuberculosis*

Generally, all *Y. pseudotuberculosis* strains are biochemically homogeneous except for production of acid from melibiose, raffinose, and salicin. *Y. pseudotuberculosis* heat-stable

somatic antigens are also used to subgroup the species. At present there are six serogroups represented by Roman numerals I-VI. Serogroups I, II, III, and IV have subtypes, but antiserum to one serogroup type will cross-react with the subtype strain and vice versa. Strains belonging to serogroups II and III are lethal when fed to adult mice even though these strains do not elaborate lipase. HeLa cell-invasive strains are esculin-positive, which is contrary to findings with *Y. enterocolitica. Y. pseudotuberculosis* strains harbor a 41-48 Mdal plasmid and will autoagglutinate at 37°C. Association of yersiniosis in humans with the presence of a plasmid has been established (38).

1. **Enrichment**. Aseptically weigh 25 g sample into 225 ml PMP broth (17). Homogenize for 30 s and incubate at 4°C for 3 weeks. At 1, 2, and 3 weeks, mix enrichment well. Transfer 0.1 ml enrichment to 1 ml 0.5% KOH in 0.5% NaCl and mix for 5-10 s. Successively streak one loopful to MacConkey agar plate and one loopful to CIN agar plate. Streak one additional loopful directly from enrichment broth to one MacConkey and one CIN agar plate. Incubate agars at RT.
2. **Isolation and identification**. Continue as in E-H, above, noting biochemical differences (Table 1). Notably, *Y. pseudotuberculosis* strains are ornithine-, sorbitol-, and sucrose-negative.

[a]+ = positive after 3 days at RT, (+) = positive after 7 days at RT.
[b] Some strains of *Y. intermedia* are negative for either Simmons citrate, rhamnose, and melibiose, or raffinose and Simmons citrate.
[c] Some biotype 5 strains are negative.

[a] Based on Wauters (51).
[b] ( ) = Delayed reaction; V = variable reactions.
[c] Biotype of serotype O:3 found in Japan.

Instructions for *Yersinia* Identification Tests

Phenylalanine deaminase agar test: Add 2-3 drops 10% ferric chloride solution to growth on agar slant. Development of green color is positive test.

Indole test: Add 0.2-0.3 ml Kovacs' reagent. Development of deep red color on surface of broth is positive test.

V-P test: Add 0.6 ml **alpha**-naphthol and shake well. Add 0.2 ml 40% KOH solution with creatine and shake. Read results after 4 h. Development of pink-to-ruby red color in medium is positive test.

Pyrazinamidase test: After growth of culture on slanted pyrazinamidase agar at RT, flood 1 ml of 1% freshly prepared ferrous ammonium sulfate over slant. Development of pink color within 15 min is positive test, indicating presence of pyrazinoic acid formed by pyrazinamidase enzyme.

Pyrizinamidase test

- **Flood 1ml of 1% freshly prepared ferrous ammonium sulfate over slant. Pink color within 15 min is +**
- **(right 2 test tubes= positive, left 2 tubes = negative).**

Beta-D-Glucosidase test: Add 0.1 g 4-nitrophenyl-**beta**-D-glucopyranoside to 100 ml 0.666 M NaH_2PO_4 (pH 6). Dissolve; filter-sterilize. Emulsify culture in physiologic saline to McFarland Turbidity Standard No. 3. Add 0.75 ml of culture to 0.25 ml of test medium. Incubate at 30°C overnight. A distinct yellow color indicates a positive reaction.

APPENDIX H

Staphylococcus aureus Procedure

Direct Plate Count Method

This method is suitable for the analysis of foods in which more than 100 *S. aureus* cells/g may be expected. It conforms to the method in ref. 1.

A. Equipment and materials

1. Same basic equipment as for conventional plate count (Chapter 3).
2. Drying cabinet or incubator for drying surface of agar plates
3. Sterile bent glass streaking rods, hockey stick or hoe-shaped, with fire-polished ends, 3-4 mm diameter, 15-20 cm long, with an angled spreading surface 45-55 mm long

B. Media and reagents

1. Baird-Parker medium (M17)
2. Trypticase (tryptic) soy agar (TSA) (M152)
3. Brain heart infusion (BHI) broth (M24)
4. Coagulase plasma (rabbit) with EDTA
5. Toluidine blue-DNA agar (M148)
6. Lysostaphin (Schwartz-Mann, Mountain View Ave., Orangeburg, NY 10962)
7. Tryptone yeast extract agar (M165)
8. Paraffin oil, sterile
9. 0.02 M phosphate-saline buffer (R61), containing 1% NaCl
10. Catalase test (R12)

C. Preparation of sample (see Chapter 1).

D. Isolation and enumeration of *S. aureus*

1. For each dilution to be plated, aseptically transfer 1 ml sample suspension to 3 plates of Baird-Parker agar, distributing 1 ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml). Spread inoculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates). If inoculum is not readily adsorbed, place plates upright in incubator for about 1 h. Invert plates and incubate 45-48 h at 35°C. Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*. Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency

when touched with inoculating needle. Occasionally from various foods and dairy products, nonlipolytic strains of similar appearance may be encountered, except that surrounding opaque and clear zones are absent. Strains isolated from frozen or desiccated foods that have been stored for extended periods frequently develop less black coloration than typical colonies and may have rough appearance and dry texture.

2. Count and record colonies. If several types of colonies are observed which appear to be *S. aureus* on selected plates, count number of colonies of each type and record counts separately. When plates of the lowest dilution contain <20 colonies, these may be used. If plates containing >200 colonies have colonies with the typical appearance of *S. aureus* and typical colonies do not appear at higher dilutions, use these plates for the enumeration of *S. aureus*, but do not count nontypical colonies. Select > 1 colony of each type counted and test for coagulase production. Add number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of *S. aureus*/g of food tested.

E. Coagulase test

Transfer suspect *S. aureus* colonies into small tubes containing 0.2-0.3 ml BHI broth and emulsify thoroughly. Inoculate agar slant of suitable maintenance medium, e.g., TSA, with loopful of BHI suspension. Incubate BHI culture suspension and slants 18-24 h at 35°C. Retain slant cultures at room temperature for ancillary or repeat tests in case coagulase test results are questionable. Add 0.5 ml reconstituted coagulase plasma with EDTA (B-4, above) to the BHI culture and mix thoroughly. Incubate at 35°C and examine periodically over 6 h period for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*. Partial clotting, formerly 2+ and 3+ coagulase reactions, must be tested further (4). Test known positive and negative cultures simultaneously with suspect cultures of unknown coagulase activity. Stain all suspect cultures with Gram reagent and observe microscopically. A latex agglutination test (AUREUS TEST™, Trisum Corp., Taipei, Taiwan) may be substituted for the coagulase test if a more rapid procedure is desired.

F. Ancillary tests

1. Catalase test. Use growth from TSA slant for catalase test on glass slide or spot plate, and illuminate properly to observe production of gas bubbles.

2. Anaerobic utilization of glucose. Inoculate tube of carbohydrate fermentation medium containing glucose (0.5%). Immediately inoculate each tube heavily with wire loop. Make certain inoculum reaches bottom of tube. Cover surface of agar with layer of sterile paraffin oil at least 25 mm thick. Incubate 5 days at 37°C. Acid is produced anaerobically if indicator changes to yellow throughout tube, indicating presence of *S. aureus*. Run controls simultaneously (positive and negative cultures and medium controls).

3. Anaerobic utilization of mannitol. Repeat 2, above, using mannitol as carbohydrate in medium. *S.aureus* is usually positive but some strains are negative. Run controls simultaneously.

4. Lysostaphin sensitivity. Transfer isolated colony from agar plate with inoculating loop to 0.2 ml phosphate-saline buffer, and emulsify. Transfer half of suspended cells to another tube (13 x 100 mm) and mix with 0.1 ml phosphate-saline buffer as control. Add 0.1 ml lysostaphin (dissolved in 0.02 M phosphate-saline buffer containing 1% NaCl) to original tube for concentration of 25 µg lysostaphin/ml. Incubate both tubes at 35°C for not more than 2 h. If turbidity clears in test mixture, test is considered positive. If clearing has not occurred in 2 h, test is negative. *S. aureus* is generally positive.
5. Thermostable nuclease production. This test is claimed to be as specific as the coagulase test but less subjective, because it involves a color change from blue to bright pink. It is not a substitute for the coagulase test but rather is a supportive test, particularly for 2+ coagulase reactions. Prepare microslides by spreading 3 ml toluidine blue-deoxyribonucleic acid agar on the surface of each microscope slide. When agar has solidified, cut 2 mm diameter wells (10-12 per slide) in agar and remove agar plug by aspiration. Add about 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures used for coagulase test to well on prepared slide. Incubate slides in moist chamber 4 h at 35°C. Development of bright pink halo extending at least 1 mm from periphery of well indicates a positive reaction.

G. Some typical characteristics of 2 species of staphylococci and the micrococci, which may be helpful in their identification, are shown in Table 1.

APPENDIX I

STEC Procedure

Procedure Outline 5B.1 Introduction 5B.2 Safety Precautions 5B.3 Equipment, Reagents and Media 5B.3.1 Equipment and Materials 5B.3.2 Media and Reagent 5B.4 Quality Control 5B.4.1 General 5B.4.2 Sample Enrichment Controls 5B.4.3 BAX® Real-time PCR Controls 5B.4.4 IMS Plating Controls 5B.5 Sample Preparation and Primary Enrichment 5B.6 Screening Procedure using BAX® Real-time PCR 5B.6.1 Procedure 5B.6.2 Interpretation of Results 5B.7 Isolation Procedure 5B.7.1 Immunomagnetic Separation and Culture Plating 5B.8 Identification and Confirmation 5B.8.1 Presumptive PCR Assay 5B.8.2 Serological Agglutination and Confirmation PCR Procedure 5B.9 Culture Storage 5B.10 Selected References

5B.1 Introduction

Shiga toxin-producing Escherichia coli strains (STEC) of various serotypes have become an increasing public health concern since E. coli O157:H7 was first identified in 1982. STEC has been implicated in numerous outbreaks including development of hemolytic uremic syndrome (HUS) in some patients. Although E. coli O157:H7 has been most commonly identified as the cause of STEC infection, isolation of non-O157 STEC strains from clinical cases, outbreaks and environmental sources has been increasing (Posse et al., 2008). A study at the Centers for Disease Control and Prevention showed that from 1983-2002 approximately 70% of non-O157 STEC infections in the United States were caused by strains from one of six major serogroups, including O26, O45, O103, O111, O121 and O145 (Brooks et al., 2005). Virulence factors for non-O157 STEC include, but are not limited to, production of the shiga-like toxins 1 and/or 2 (Stx1, Stx2) and intimin (eae). Cattle and other ruminants appear to be the main reservoir of non-O157 STEC, as well as the O157:H7 serotype (Arthur et al., 2002). With carriage rates of non-O157 STEC in cattle being a public health concern, a method was devised to detect and isolate the six major non-O157 STEC serogroups (O26, O45, O103, O111, O121 and O145) in ground beef and beef trim. This method utilizes the BAX® STEC suite followed by cultural isolation. The BAX® System Real-time PCR Screening Assay for stx and eae detects the presence of the shiga toxin (stx) and intimin (eae) genes. Note that while this assay detects shiga toxin gene sequences, it does not differentiate between stx1 and stx2. Two additional BAX® real-time PCR assays, STEC Suite Panel 1 and Panel 2, are used to identify genes within the O antigen gene cluster specific for each serogroup. Cultural isolation of non-O157 STEC from screenpositive enrichments (positive for stx, eae and top six O antigen gene cluster) proceeds using immunomagnetic separation (IMS) beads coated with serogroup-specific antibodies followed by plating onto mRBA. A post-IMS acid treatment step is performed to help reduce background microflora that grow on mRBA. Many strains of STEC have been reported to have acid tolerance at pH 2 while competitor organisms show pH sensitivity (Grant, 2004; Bagwhat et al., 2005). Colonies on mRBA are tested for the presence of O antigens specific for the top six STEC serogroups using an agglutination test. Agglutination positive colonies are then streaked onto tryptic soy agar with 5% sheep blood (SBA) for confirmation using BAX® real-time PCR assays and biochemical identification.

5B.2 Safety Precautions

Similar to E. coli O157:H7, non-O157 STEC serotypes are human pathogens with a low infectious dose. The use of gloves, protective laboratory coats and eye protection is for all post enrichment viable culture work. Work surfaces must be disinfected prior to and immediately after use. Laboratory personnel must abide by CDC guidelines for manipulating Biosafety Class II pathogens. A Class II laminar flow biosafety cabinet is recommended for activities with potential for producing aerosols of pathogens. All available Safety Data Sheets (SDS) shall be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the materials should read all SDS.

5B.3 Equipment, Reagents and Media

5B.3.1 Equipment and Materials

a. Balance, sensitivity ± 0.1 g b. Blending/mixing equipment: Paddle blender, Sterile Osterizer-type blender with sterilized cutting assemblies, and blender jars or equivalent and adapters for use with Mason jars c. Sterile plain, clear polypropylene bags (ca. 24" x 30 - 36"), or Whirl-Pak□ type bags (or equivalent) d. Incubators, static 42 ± 1°C and 35 ± 2°C e. PCR tube holder (Qualicon or equivalent). f. Cell lysis tube cooling block (Qualicon or equivalent) held at 5 ± 3ºC g. PCR cooling block (Qualicon or equivalent) held at 5 ± 3ºC h. Heating block set at 37 ± 2ºC i. Heating block set at 95 ± 3ºC j. Repeating pipettor to deliver 200 ± 20 µl and sterile tips k. Pipettor to deliver 20 ± 1µl, and sterile disposable filtered tips l. Pipettor to deliver 150 ± 15 µl, and sterile disposable filtered tips m. Eight-channel pipettor to deliver 30 ± 3 µl, and sterile disposable tips n. Pipettor to deliver 5 ± 1 µl, and sterile disposable tips. o. 12 X 75 mm Falcon 352063, or equivalent, tubes p. Cell lysis tubes and caps, cell lysis tube rack and box (Genemate□ 8 strip tubes, ISC Bioexpress, T-3120-5 or equivalent) q. Pipettor or pipettes to deliver 5 ml r. Dupont Qualicon BAX□ System Q7 Instrument s. BAX® System Real–time PCR Assay STEC Screening (Part # D14642964) held at 5 ± 3ºC t. BAX® System Real–time PCR Assay STEC Panel 1 (Part # D14642970) held at 5 ± 3ºC u. BAX® System Real–time PCR Assay STEC Panel 2 (Part # D14642987) held at 5 ± 3ºC v. Micropipettors for culture plating to deliver volumes ranging from 15-1000 µl with sterile disposable filtered tips w. VITEK® 2 system x. GN cards for VITEK® 2 system (bioMerieux Vitek, Inc.) y. Heating block (95-99°C) or thermocycler for DNA preparation step) z. Vortexer aa. Centrifuge that holds microcentrifuge tubes and is capable of speeds up to 16,000 x g bb. Centrifuge plate adapter for the centrifugation of 96-well PCR plates cc. Disposable, sterile pipettes for volumes 1.0 ml and for 5.0 ml. dd. Sterile, inoculating loops, “hockey sticks” or spreaders, and needles ee. Rotating tube agitator with clips to hold microcentrifuge tubes ff. Sterile, disposable 12 x 75 mm polypropylene or polystyrene tubes gg. Sterile microcentrifuge tubes (1.5 - 2.0 ml) hh. Sterile 50 ml conical tubes ii. Sterile 40 µm Cell Strainer jj. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02) kk. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09) ll. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03) mm. Tray, autoclavable, approximately 130 mm x 83 mm for use with the OctoMACS® nn. Sterile filter or non-filter bags oo. Optical density reader

5B.3.2 Media and Reagents

a. Modified Tryptone Soya Broth (mTSB) b. Modified Rainbow Agar (mRBA) [Rainbow® Agar O157 Biolog Inc., Hayward California, 94545] containing 5.0 mg/L sodium novobiocin,

0.05 mg/L cefixime trihydrate and 0.15 mg/L potassium tellurite c. Cefixime trihydrate d. Tryptic soy agar with 5% sheep blood [Sheep Blood Agar (SBA)] e. 1.0 N Hydrochloric Acid (HCl) f. Physiological saline solution (0.85% NaCl) g. 1X Tris-EDTA (TE) Buffer h. E Buffer, approximately 7 ml per sample (See Media and Reagents Appendix 1, Buffered Peptone Water, Bovine Albumin Sigma and Tween-20®) i. Disinfectant (Lysol® I. C., 2.0%) j. Romer Labs RapidChek® CONFIRM STEC Immunomagnetic Separation (IMS) Kit with anti-O26 antibody-coated paramagnetic beads, anti-O103 antibody-coated paramagnetic beads, anti-O111 antibody-coated paramagnetic beads, anti-O145 antibody-coated paramagnetic beads, anti-O45 antibodycoated paramagnetic beads, and anti-O121 antibody-coated paramagnetic beads k. RNase free, DNase free PCR Certified Water l. Biochemical test kit and system, GN cards (VITEK® 2 system, bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395) m. Abraxis non-O157 STEC Latex Agglutination Test (LAT) Kits or equivalent specific for serogroups O26, O45, O103, O111, O121 and O145

5B.4 Quality Control

5B.4.1 General

a. Unless otherwise stated, weight and volume ranges and minutes have a tolerance of ±2%. b. All media, plates and buffers shall be warmed to 18-35°C prior to use. c. The top six non-O157 STEC control strains shall meet the following genetic characteristics: stx+ and eae+. Such strains can be obtained through reference culture collection centers including but not limited to the American Type Culture Collection (ATCC), the STEC Center at The Michigan State University and the E. coli Reference Center at The Pennsylvania State University. Non-O157 strains (stx+, eae+) must be used by FSIS Laboratories to prepare the DNA template positive PCR control. However, for safety considerations, toxin-attenuated or toxin-negative strains that have an appearance on mRBA typical of the non-O157 STEC may be used as controls on plating media for serological agglutination testing. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses. The following non-O157 STEC control strains shall be used when stated in the method: i. E. coli O26, which shall be stx positive and eae positive ii. E. coli O45, which shall be stx positive and eae positive iii. E. coli O103, which shall be stx positive and eae positive iv. E. coli O111, which shall be stx positive and eae positive v. E. coli O121, which shall be stx positive and eae positive vi. E. coli O145, which shall be stx positive and eae positive Note: In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses.

5B.4.2 Sample Enrichment Controls

Include with each sample batch, a positive growth control (E. coli O157:H7 strain 465-97 or other reference strain that is stx-, eae+) inoculated into a meat matrix free of the target analyte, and an uninoculated media (mTSB) control.

5B.4.3 BAX□ Real-time PCR Controls

a. stx/eae screen PCR • 20 µl enrichment from bioluminescent E. coli O157:H7 strain 465-97 (growth control) • DNA template (5 µl) from a cocktail of top six STEC cultures (PCR positive control) • Uninoculated mTSB medium (20 µl) b. Serogroup-specific screen PCR (Panel 1 and Panel 2) • DNA template (5 µl) from a cocktail of top six STEC cultures (PCR positive control) •

Uninoculated mTSB medium (20 µl) c. Optional stx/eae presumptive PCR / stx/eae confirmatory PCR • DNA template (5 µl) from a cocktail of top six STEC cultures (PCR positive control) d. Optional serogroup-specific presumptive PCR (Panel 1 and Panel 2) / Serogroup-specific confirmatory PCR (Panel 1 and Panel 2) • DNA template (5 µl) from a cocktail of top six STEC cultures (PCR positive control) To prepare PCR positive control DNA template, FSIS laboratories shall grow the top six STEC cultures on SBA and incubate at 35±2°C for 16-24 h. Colonies shall be used to create a culture suspension in PCR certified water corresponding to approximately 109 CFU/ ml. In one tube, 1.0 ml from each suspension shall be added to 4.0 ml of PCR certified water to create a 10.0 ml cocktail of all six strains. This will provide approximately a 108 CFU/ml cocktail using each strain. One hundred microliter aliquots of the suspension are then transferred to PCR tubes or microcentrifuge tubes and heated at 95-99°C for 10 minutes on a thermocycler or heating block. The tubes shall be centrifuged at 10,000 x g for 3 minutes to pellet cellular debris. The supernatant shall be used as the PCR positive control for all PCR assays. DNA control template can be prepared as a batch, transferred to smaller volume tubes, and stored at ≤ -20°C for 1 year.

5B.4.4 IMS Plating Controls

Streak an isolate from the serogroup(s) of interest (based on serogroup-specific PCR results) onto mRBA and incubate along with the samples that have been treated with the IMS procedure.

5B.5 Sample Preparation and Primary Enrichment

Note: Disinfect the sample package prior to opening. a. For raw beef, raw beef mixes, beef trim, and trim components, place the 325 ± 32.5g test portion per submitted sample into the sterile bag with mesh filter. Ensure that the entire test portion is on the same side of the mesh filter. Add 975 ± 19.5 ml of mTSB to the test portion to provide a 1:4 dilution (one portion of product to three portions of broth). Pummel, blend or hand massage until well mixed. Incubate the test portion and the enrichment media at 42±1°C for 15-24 hours. Each group of samples should include a positive control enrichment (E. coli O157:H7 strain 465-97) and an uninoculated enrichment medium control. b. For environmental sponges and carcass sponges with 10 ml of buffer, add 50 ± 5 ml of mTSB broth. For carcass sponges with more buffer, use a 1:6 ratio of mTSB (for example, a swab with 25 ml of buffer will use 125 ml of enrichment broth) to each bagged sponge sample. Pummel, blend or hand massage until well mixed.

5B.6 Screening Procedure using BAX□ Real-time PCR

5B.6.1 Procedure

Following incubation, perform the rapid screen using 20 µl of mTSB sample enrichment for all matrices except raw beef mixes containing poultry. Follow the current BAX□ System User's Guide for preparing reagents, performing the STEC screening PCR, Panel 1 and Panel 2 PCR, and interpreting results, if applicable. The real-time PCR assay developed for the ABI 7500 FAST is an alternative screen described in MLG 5B Appendices 1 and 3. Following incubation of raw beef mixes containing poultry, a centrifuge step must be performed prior to BAX® screening: • Dispense 200 ± 20 µl lysis reagent to each cell lysis tube. • Heat the filled lysis tubes for 20 ±1 minute at 37 ± 2°C. Aseptically transfer 1 ml of the poultry mix enrichment sample to a sterile 1.5 ml microcentrifuge tube. • Centrifuge at a setting of 1,500 x g for 1 minute (at speed) to pellet large debris. Supernatant will still not be clear at this low speed but should no longer

have large particles of meat suspended. • Transfer the supernatant to a new sterile 1.5 ml microcentrifuge tube. It is essential to ensure that none of the pelleted debris is carried over with the supernatant. • Centrifuge supernatant at 10,000 x g for 5 minutes. • Discard the supernatant from the centrifuge tube, leaving a little of the supernatant if necessary so the pellet is not disturbed during this step. • Suspend the pellet in 100 µl of PCR grade water either by vortexing or using the pipet tip. • Add 5 µl of the suspension directly to the pre-heated lysis buffer that was prepared during the initial steps. • Heat the inoculated lysis tubes for 10 ± 1 minute at 95 ± 3 °C. Perform remainder of the PCR test according to manufacturer's instructions.

5B.6.2 Interpretation of Results

a. Samples that test negative for the BAX□ STEC screening PCR (stx, eae) shall be reported as negative. Samples that test positive for the STEC screening PCR (stx, eae) will be further analyzed by using the positive BAX□ lysate in the Panel 1 and Panel 2 tests. Samples must remain chilled at 2-8°C until loaded into the instrument. Remaining lysate may be sealed and stored for additional testing with other BAX® System STEC suite assays. Lysates may be stored at 2-8°C for up to 7 days or at -20 ± 3°C for up to 14 days. Note: For Panel 1 and Panel 2 results, each well must be clicked individually and the results for each individual O-group should be recorded. b. Samples that test positive for the STEC screening PCR (stx, eae) but negative for both Panel 1 and Panel 2 shall be reported as negative. If any of the Ogroups from Panel 1 or Panel 2 are positive, the sample shall be reported as a potential positive. Proceed with the isolation procedure as described in Section 5B.7. c. Samples that are indeterminate or have an invalid result for the BAX□ STEC screening PCR (stx, eae) should be tested again using STEC screening PCR and Panels 1 and 2 assays using either the same lysate or preparing new lysate tubes. Samples that are BAX□ STEC screening PCR (stx, eae) positive but indeterminate or have an invalid result on one or both Panel 1 and 2 assays proceed to Section 5B.7 Isolation Procedure and analyze for the indeterminate O groups. Alternatively, the laboratory may review the cause and perform a correction. Based on the findings, the laboratory may: • repeat the BAX□ analysis from the rack loading step or • prepare new BAX□ tubes and repeating the analysis. d. In analytical runs where the positive control tests BAX□ -negative, indeterminate, or has a signal-error result, the entire batch of samples is affected and a review of the cause and a correction shall be performed. Based on the findings the laboratory may: • repeat the BAX□ analysis from the rack loading step • prepare new BAX□ tubes and repeating the analysis • analyze all of the samples culturally. If reanalysis of a sample with indeterminate or invalid BAX□ results is unsuccessful then use the alternative screen, perform cultural analysis, prepare fresh analytical portions from the sample reserve, or discard the sample.

5B.7 Isolation Procedure

Samples that are potentially positive by PCR screen results shall be plated onto mRBA following IMS. In the isolation procedure, IMS beads shall be used for the specific serogroup identified by the serogroup PCR reaction (i.e. anti-O26 will be used for samples with screen results positive for O26, anti-O45 for O45 PCR positive reactions, anti-O103 for O103 PCR positive reactions or anti-O121 for O121 PCR positive reactions, anti-O111 for O111 PCR positive reactions and/or anti-O145 for O145 PCR positive reactions). A postIMS acid treatment step has been added to reduce background flora on the mRBA plate. Following the one hour acid treatment step, samples are diluted 1:1 with E-buffer and 0.1 ml is spread plated onto mRBA. Additionally, the suspension is diluted 1:10 and 0.1 ml is spread plated onto mRBA.

5B.7.1 Immunomagnetic Separation and Culture Plating

a. Remove mRBA plates from 2-8°C storage, allowing 4 plates for each screenpositive culture and one plate for each serogroup control strain. Be sure that plates have no visible surface moisture at the time of use. If necessary, dry plates (e.g. for up to 30 minutes in a laminar flow hood with the lids removed) prior to use. Dried plates that are not used should be labeled "dried", placed in bags and returned to 2-8°C. b. For each screen-positive culture, label two sterile microcentrifuge tubes (for step d and step m), one 50 ml conical centrifuge tube (for step c) and four 12 x 75 mm capped tubes (for steps i and j). For three of the 12 x 75 mm tubes, add 0.9 ml E-Buffer and label one tube as 1:10, one tube as 1:100 and one tube as acid 1:10. c. Sample preparation from overnight enrichment: For each serogroup that the sample is positive, transfer approximately 2-5 ml from overnight enrichment through a 40 µm Cell Strainer into a 50 ml conical centrifuge tubes. d. Binding of paramagnetic antibody beads to specific serogroup: Transfer 50.0 µl (or volume recommended by the manufacturer) of appropriate immunomagnetic capture beads determined by the serogroup PCR screen results (O26, O45, O103, O111, O121 or O145) to a sterile, labeled microcentrifuge tube. Next, add 1 ml of enrichment filtrate to the appropriately labeled tube. e. Place the microcentrifuge tubes containing enrichments and capture beads on LabQuake® Agitator and rotate tubes for 15 minutes at 18-30°C (or time recommended by the manufacturer). f. For each sample, place one MACS® Large Cell Separation Columns onto the OctoMACS® Separation Magnet. Fill the tray below the separation magnet with disinfectant. Prime each separation column with at least 0.5 ml of Ebuffer and allow the liquid to pass completely through before adding sample. g. Binding of beads to magnetic columns: Once the liquid has passed through the column, add the 1.0 ml of enrichment plus IMS beads to each appropriately labeled column and allow liquid to completely pass through. h. Wash steps (4X): Add 1.0 ml of E-buffer to each column allowing the liquid to pass completely through. Repeat 3 more times for a total of 4 washes. i. Elution step: After the last wash has drained, remove the column from the OctoMACS® Magnet and insert the tip into an empty labeled 12 x 75 mm tube. Apply 1.0 ml of E Buffer to the column, and using the plunger supplied with the column, immediately flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes. Repeat this for each column. j. Make a 1:10 dilution of each treated bead suspension by adding 0.1 ml of the bead suspension to a 12 x 75 mm labeled tube containing 0.9 ml E-Buffer. Make a 1:100 dilution by adding 0.1 ml of the 1:10 dilution to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer. k. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (1:10 dilution and 1:100 dilutions) onto a labeled mRBA plates. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate. l. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C. m. Acid Treatment: For each sample, transfer 450 µl of the undiluted bead suspension (MACS column eluant) to an empty labeled microcentrifuge tube. Add 25 µl of 1N hydrochloric acid (HCl) to this bead suspension and vortex briefly. This will bring the pH to 2.0-2.5 using E-buffer. n. Place the microcentrifuge tubes containing the acid treated suspension on a LabQuake® Agitator and rotate tubes for 1 hour at 18-30°C temperature. o. After 1 hour, dilute the suspension by adding 475 µl of E-buffer. p. Vortex briefly to maintain beads in suspension and plate 0.1 ml of the neutralized suspension onto a labeled mRBA plate. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate. q. Add 0.1 ml of the suspension to a labeled tube containing 0.9 ml E-buffer and vortex briefly. This shall represent a 1:10 dilution of the acid-treated cell suspension. Plate 0.1 ml of the diluted suspension onto an appropriately

labeled mRBA plate. r. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C.

5B.8 Identification and Confirmation

Following 20-24 h incubation of mRBA, plates will be examined for colonies that agglutinate with latex agglutination reagents specific for the serogroup of interest. Colony colors from representative strains of each serogroup are listed in MLG 5B Appendix 2 Morphologies of Representative Strains from Top Six non-O157 Shiga Toxin-Producing Escherichia coli (STEC) Grown on mRBA. However, the coloration of colonies described in MLG 5B Appendix 2 may vary based on proximity to other competitor colonies or medium discoloration due to competitor colony growth. Since the morphologies of the targeted STEC colonies may vary widely among strains and serogroups, test at least one colony from each identified colony morphology found on the mRBA plate. Samples that have no growth or only contain agglutination negative colonies on mRBA are negative for non-O157 STEC. Any sample with agglutination positive colonies for the serogroup of interest is a presumptive positive for non-O157 STEC. Agglutination positive colonies shall be streaked onto SBA for confirmation on the following day. Following a restreak of presumptive colonies and 16-24 h incubation of the SBA, agglutination-positive colonies shall be confirmed with BAX□ real-time PCR and biochemical identification. The confirmatory BAX□ real-time PCR shall include the Screening assay (stx and eae) and the O-group Panel which includes the serogroup that the colony had a positive agglutination reaction (i.e. Panel 1 for O26, O111, O121, and Panel 2 for O45, O103, and O145). If no colony picks isolated from the mRBA confirm by PCR and VITEK® 2, the sample is negative for non-O157 STEC. If a FSIS Laboratory has confirmatory test results insufficient to allow identification (i.e. confirmatory PCR positive but biochemically negative), then the isolate is transferred to the Outbreaks Section of the Eastern Laboratory Microbiology Branch (OSEL), or current FSIS reference laboratory, for further testing prior to reporting.

5B.8.1 Presumptive PCR Assay

A PCR test may be performed directly on agglutination positive colonies from the mRBA to verify presumptive positive colonies using the following procedure. The presumptive PCR assay is optional for non-FSIS laboratories. a. Transfer the remainder of an agglutination positive colony from the mRBA plate into 50 µl of Molecular Grade Water (for up to 5 colonies). b. Add 5 µl from the suspension to a BAX□ lysis tube. Heat lysate at 37 ± 2o C for 20 ± 1 minute then 95 ± 3o C for 10 ± 1 minute then cool for 5-30 minutes in cooling block. Add 30 µl of the lysate to the BAX□ Real-time Screening assay (stx/eae) reaction tube and the appropriate Panel reaction tube each on a cooling block. Note: Each PCR assay shall include a positive control as described in Quality Control section 5B.4. c. The sample is considered negative if any of the 3 PCR targets (stx, eae or serogroup) are negative. d. If an agglutination positive colony from mRBA is positive for O group, stx and eae targets, the sample is considered a presumptive positive for non-O157 STEC. Refer to section 5B.8.2 for confirmation of the isolates as non-O157 STEC. e. From the previous suspension, streak SBA for isolation. Incubate inoculated SBA plates at 35 ± 2°C for 16-24 hours.

5B.8.2 Serological Agglutination and Confirmation PCR Procedure

a. Use an inoculating loop or needle to transfer a portion of an isolated colony from the mRBA plate to serological agglutination reagent. Follow manufacturer’s instructions on procedure and

interpretation. Control Reactions: A reference strain from the serogroup of interest plated on mRBA shall be used as the positive culture control. For presumptive PCR screen from colonies isolated on mRBA, refer to section 5B.8.2 Presumptive PCR Assay. b. Transfer the remainder of an agglutination positive colony from the mRBA plate onto SBA for further biochemical and genetic confirmation. Streak up to 5 agglutination positive colonies onto SBA plates. Incubate plates at 35 ± 2°C for 16-24 hours. c. Following SBA incubation, perform the agglutination test again on colonies from the SBA plate. d. To confirm agglutination-positive colonies using BAX□ real-time PCR, prepare a template by suspending an agglutination positive colony from the SBA plate in 50 µl of Molecular Grade Water and adding 5 µl of this suspension to BAX□ lysis buffer. e. Continue with the BAX□ system protocol from the "Perform Lysis" step. The BAX□ lysate will then be used for the STEC Screening assay and the appropriate Panel assay Note: Each PCR assay shall include a positive control as described in Quality Control section 5B.4. f. Additionally, perform biochemical identification (VITEK® 2) on agglutination positive colonies from the incubated SBA. A positive isolate shall be identified biochemically as E. coli. g. If the isolate is agglutination positive for top six STEC serogroups, BAX□ real-time PCR positive for stx, eae, and top six serogroup genes and biochemically identified as E. coli, the sample is positive for non-O157 STEC. h. If the isolate and any additional colony picks from mRBA are ultimately determined to be BAX□ real-time PCR negative for stx, eae, top six serogroup genes, the sample is negative for non-O157 STEC.

5B.9 Culture Storage

For storage requirements of the fluorescent E. coli O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain) refer to MLG 5 Detection, Isolation and Identification of Escherichia coli O157:H7 from Meat Products, Section 5.3.c. Store other "working" non-O157 E. coli stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at 35± 2°C, and then store them at 2-8°C. Use one of the slants as the working culture. Use the other slant for subculturing to reduce the opportunity for contamination. For long term storage, freeze cultures using cryo-beads, i.e., Cryostor™ or lyophilize.

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APPENDIX J

Type A Swine Influenza Procedure

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Real-Time RT-PCR for the Detection of Type A Swine Influenza Virus and Identification of A Novel N1 Subtype in Clinical Samples

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Real-Time RT-PCR for the Detection of Type A Swine Influenza Virus and Identification of A Novel N1 Subtype in Clinical Samples

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6.1. Panigrahy, B., Senne, D.A., Pedersen, J.C., Avian Influenza virus subtypes inside and outside the live bird markets, 1993-2000: a spatial and temporal relationship. Avian Dis. 46:298-307.
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6.6. SOP-AV-0007, Evaluation and Quality Control Testing of Real-Time PCR Primers and Probes
6.7. SOP-AV-0028, RNA Extraction Using a Magnetic Particle Processor
6.8. SOP-BPA-0023, Swine Influenza Virus Isolation
6.9. WI-DVL-0004, Work Instruction for analysis of controls for NAHLN AI, APMV-1, and SIV real-time PCRs
6.10. SOP-BPA-0023, Swine Influenza Virus Isolation

7. Revision History

8. Appendices

1. Purpose/Scope

1.1. Background

The real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) procedure described in this protocol utilizes a one step procedure with specific primers designed to amplify a portion of the genome that contains a target PCR sequence. Non-extendible fluorogenic hydrolysis/TaqMan probes monitor the target PCR product formation at each cycle during the PCR reaction. The probes are labeled at the 5' end with a reporter dye (e.g. FAM) and non-fluorescing quencher (e.g. blackhole quencher [BHQ-1]) at the 3' end. The proximally located quencher absorbs the emission of the reporter dye via fluorescence resonance energy transfer (FRET). Following hybridization of the probe to the target, the 5' nuclease activity of Taq polymerase will cause hydrolysis of the probe, separating the quencher from the reporter dye. This separation results in unquenched fluorescence emission of the reporter dye, which is detected spectrophotometrically and recorded. The amount of fluorescence recorded and the cycle number of detection are proportional to the amount of target template in the samples.

The swine influenza virus (SIV) rRT-PCR procedures described in this protocol have two unique primer/probe combinations; one that targets the matrix gene and one that is subtype specific for the N1 of the 2009 lineage H1N1 influenza virus first identified in the United States in humans in April 2009. The matrix primer/probe set is designed to detect influenza A viruses. The matrix procedure is a quasi-multiplex rRT-PCR which uses a single forward primer and probe in conjunction with two reverse primers. It should be noted that this virus has undergone genetic reassortment resulting in the N1 gene being found in viruses other than the 2009 H1N1 SIV.

Tissues or swabs from more than one animal are not pooled together. It should be emphasized that the rRT-PCR technique will detect viral nucleic acid from infectious as well as noninfectious virus. The rRT-PCR is not the test of choice to determine if infectious SIV is present in environmental samples. It is recommended that environmental samples be tested for detection of live virus by isolation.

Robotic 96-well and 24 specimen RNA extraction procedures and 96-well amplification platforms have been included in the protocol for high throughput processing and testing. Equivalency studies performed with avian influenza virus have demonstrated the Thermo® KingFisher, Qiagen® BioSprint , and MagMAX™ Express instruments are equivalent for the isolation of RNA from swab specimens using the Ambion® MagMAX™ Viral RNA Isolation Kit (SOP-AV-0028). No equivalency studies have been conducted to support the use of a 384-well amplification format.

The procedures described are used in the Diagnostic Virology Laboratory (DVL) of the National Veterinary Services Laboratories (NVSL). The brands of equipment and extraction reagents listed in the protocol have been validated for use with the United