

**Title:** An evaluation of the suitability of porcine lung tissue for human consumption -**NPB #13-250**

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### Industry Summary

The objective of this study was to provide evidence of the safety of pork lungs for human consumption via determining the prevalence of potentially pathogenic bacteria and infectious agents known to be prevalent in pork. Specifically, the goal was to collect evidence that could be used to petition the current regulation disallowing saving 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. With this said, it is believed that there is profit potential in saving pork lungs and exporting them to the specified countries. Pork lungs must first be deemed safe and edible before they can be sold on the export market.

Lungs were collected from a total of 6 federally inspected pork processing facilities that were specific to killing either young market barrows/gilts or sows. 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. All of the lungs collected were removed and processed using aseptic techniques to prevent any exogenous contamination.

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The lung samples collected were tested for the prevalence of specific pathogens and other physical contamination. The lungs did not test positive for *Yersenia* spp., *Influenza*, or *Mycobacterium* spp., and they contained low yeast and mold counts. However, multiple samples that were 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 physical contamination within the airways of the lungs. These results suggest that pork lungs are not safe and should not be saved for human consumption.

### **Keywords**

Pork, Lungs, Offal, E. coli, Salmonella, Pathogens

### **Scientific Abstract**

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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 material within the airways of the lungs. These results suggest that pork lungs are not safe and should not be saved 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 all of 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 the markets exists. However, USDA-FSIS regulation prohibits 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 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. In this document, it was specifically stated that several hundred BEEF lungs were evaluated by trained pathologists, and it was reported that 93.5 % of these 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. This includes the PI’s included on this proposal reaching out to FSIS EIAO personnel, FSIS Deputy District Managers, and personnel from the FSIS Policy

Development Division. 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 is the objective of the proposed research to provide evidence of the safety of porcine lungs for human consumption via determining the prevalence of potentially pathogenic bacteria and infectious agents known to be prevalent in pork. This includes determining the prevalence of bacterial pathogens including the seven predominant STECs, *Salmonella* spp., and *Campylobacter* spp. Additionally, discussions with USDA have indicated that pneumonia and specifically tuberculosis may be of great concern to USDA-FSIS, when considering allowing lungs for human consumption. Therefore, the prevalence of *Mycobacterium bovis*, *M. tuberculosis*, *M. avium*, and *Influenza* will be evaluated by the proposed work. 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* sp. in domesticated porcine lungs is unlikely due to modern production practices, specifically confinement production practices. To this point, *Mycobacterium* sp. 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. 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* sp. associated with tuberculosis will be cultured from the lungs of domestic swine. Additionally, most all sources, including USDA, agree that tuberculosis is not a foodborne disease and over 90 % of healthy youth and adults are immune; however, exposure to animals infected with *M.bovis* and *M. tuberculosis* can cause tuberculosis in humans. In addition, histopathology examination of lungs will be performed to understand the prevalence of lesions, pneumonia, abscesses, and other contaminants including dust and dirt. To directly address the concerns of USDA dating back to 1969, the prevalence of yeasts and molds will also be determined.

## **Objectives**

The objective of the proposed research is to provide evidence of the safety of porcine lungs for human consumption via determining the prevalence of potentially pathogenic bacteria and infectious agents known to be prevalent in pork. Specifically, evidence will be gathered to petition the current regulation disallowing saving of porcine lungs for human food. This includes determining the prevalence of bacterial pathogens including the seven predominant STEC's, *Salmonella* spp., and *Campylobacter* spp. Additionally, the prevalence of *Mycobacterium bovis*, *M. tuberculosis*, *M. avium*, and *Influenza* will be evaluated by the proposed work. Also, histopathology examination of lungs will be performed to understand the prevalence of lesions, abscesses, and other contaminants including dust and dirt. Ultimately, in the event that the results are in support of the fact that porcine lungs are safe for human consumption, these data will be summarized in the form of a petition to USDA-FSIS to change or suspend regulation that prohibits porcine lungs from being consumed as human food.

## **Materials and Methods**

This document is a summary of results for the project sponsored by the National Pork Board and conducted by Colorado State University investigating the suitability of porcine lung tissue for human consumption. The names of the packing plants and producers involved will be kept confidential throughout the entire course of the study and therefore will not be mentioned in any final data.

### *Pork Lung Collection Process*

Lungs were collected from a total of 6 federally inspected pork processing facilities, 4 commercial pork processing plants that were harvesting youthful, market weight gilts and barrows and 2 commercial pork processing plants that were harvesting mature sows. 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. In an attempt 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. The 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 was a composite consisting of 5 randomly selected lungs except for the 48 samples collected for histopathological examination. These 48 samples each represented one lung.

#### *Processing and Shipping the Pooled Samples for Pathogenic Testing*

Lung samples evaluated for microbiological testing were pooled samples representing 5 randomly selected pork lungs. Each pooled sample consisted of approximately 500 grams of lung tissue representing 5 animals. The pieces were removed from the lungs using sterilized scissors and scalpels. From each individual lung, 100 g of lung tissue was removed from the apical lobes, middle lobe, diaphragmatic lobes, and the accessory lobe on both the right and left sides of the lung. Unlike the histology samples, there was no lymph node tissue included in the pooled samples. All of these pieces 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 cold packs and then shipped overnight to a commercial laboratory. The bags of samples were adequately insulated to prevent direct contact with the cold packs. Microbiological samples were screened by an accredited commercial laboratory for the prevalence of *Salmonella* sp., *Campylobacter*, *Staphylococcus aureus*, *E. coli* STECs, *Yersenia* spp., mold, and yeast. Paired samples were also sent to the University of Minnesota Veterinary Diagnostic Lab to be for the prevalence of *Mycobacterium*, *Streptococcus suis*, 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 consisted of 5 pieces from a single lung, which was randomly chosen along with the microbiological samples. For each lung designated for histology, samples were obtained in the processing plant from the following 5 anatomical locations (Figure 1.): 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; 5) tracheobronchial lymph node. 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 to prevent direct contact with the cold packs. Colorado State University conducted the histopathological examinations.

## *Pathogen Testing Procedures*

### *Mycobacteria Procedure*

Lung tissue samples were reduced to 2 grams and placed in a conical tube containing 35 ml of sterile water. The tube was mixed vigorously and then placed in a vortex for 30 minutes. The tube was left standing at room temperature for at least 30 minutes 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 hours. The samples were then centrifuged for 30 minutes at 900 x g. The supernatant was removed and the cellular debris pellet was suspended with 1 ml of the antibiotic brew and vortex well. Tubes were incubated again at 35-37°C overnight. Four test tubes were inoculated at room temperature with Herrold's Egg Yolk agar (3 with mycobactin and 1 without) with .250 µl of the resuspension. Samples plated on Herrald'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.

### *APC Procedure*

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 minutes. Appropriate dilutions were plated to enumerate aerobic plate counts. Petrifilms were incubated with the clear side up for 48 hours  $\pm$  3 hours at 35°C  $\pm$  1°C. APC counts were reported in CFU/g.

#### *Salmonella Procedure*

*Salmonella* was detected in the lung tissue samples using the VIDAS Easy SLM test from bioMerieux Inc.. A 25 g sample of lung tissue was enriched with 225 ml of broth and then pummeled for 2 minutes. Sample was then incubated for 16-22 hours at 35°C  $\pm$  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 minutes. The VIDAS strips were removed and cooled at room temperature for 10 minutes. Results were recorded after 45 minutes.

#### *Yeast and Mold Procedure*

The Bacteriological Analytical Manual (BAM) was referenced. 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 5 days. Samples were incubated for an additional 48 hours to allow time for heat or chemically-stressed cells and spores to grow if no growth was detected after the initial 5 days.

#### *Campylobacter Procedure*

The DuPont™ BAX® System Real Time PCR Assay for *Campylobacter* was used to determine pathogen prevalence. A 25 g sample of lung tissue was diluted by a factor of 1:10 in single-strength Bolton broth. A total of 200  $\mu$ L of lysis reagent and 5  $\mu$ L of the diluted sample were added to cluster tubes. The cluster tubes were then heated for 20 minutes at 37°C followed by 10 minutes at 95°C in a dry block heater. The cluster tubes were cooled for 5 minutes before 30  $\mu$ L of their content was transferred to PCR tubes in a cooling block. PCR tubes were placed in a PCR cycler for 90 minutes to receive the final results.

#### *Streptococcus suis Procedure*

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  $\mu$ l 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.

#### *Yersenia spp. Procedure*

The BAM was used as a reference for the *Yersenia spp.* testing procedure. 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 the enrichment was transferred to 0.1 ml of 0.5% KOH in 0.5% saline and mixed for 2-3 seconds. 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 seconds, and streaked on an additional MacConkey agar plate. Plates were incubated for 1-2 days at 30°C and examined for colonies. No suspected *Yersenia* species grew on the MacConkey agar so no additional confirmatory agars were needed.

#### *Staphylococcus aureus Procedure*

The BAM was used as a reference for the *Staphylococcus aureus* testing procedure. A 1 ml sample was distributed equally to 3 plates of Baird-Parker agar. Inoculum was spread over the surface of the agar and placed inverted in an incubator for 45-48 hours 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*

The BAM was used as a reference for the Non-O157 STEC's testing procedure. 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 hours. The samples were analyzed by a real-time PCR using the BAX<sup>®</sup> system.

### *Type A Swine Influenza Virus Procedure*

The 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. The probes are labeled with a reporter dye at one end and a non-fluorescing quencher at the other end. The amount of fluorescence that is generated and the cycle number of detection was proportional to the amount of target template

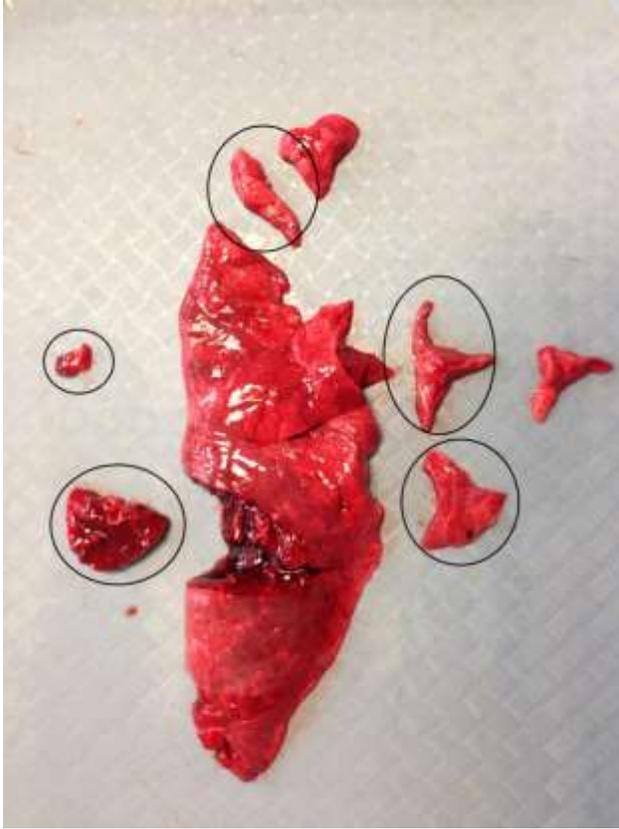


Figure 1. Anatomical locations of histology samples.

## Results

### *Pathogen data*

Samples that were collected for specific pathogen testing were pooled samples that each contained lung tissue from five hogs. Multiple 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's 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 1). All fifteen of the samples that tested positive for STEC's contained more than one Shiga toxin-producing *E. coli* and three of these samples actually contained all six of the major STEC's (Table 2). Only one sample, or 2.3% of all the samples, tested positive for *Campylobacter* (Table 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 1). *Yersenia* spp., *Influenza*, and *Mycobacterium* spp., were also tested for, however no samples tested positive for these pathogens.

#### *Histology data*

The histopathology results indicated that 25 of the 48 samples from the 48 hogs contained some aspirated material (Table 3). The aspirated material included either plant material, blood, or fluid that appeared to originate from either the mouth or the esophagus. 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. In other words, 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, the prevalence of the major pathogens that were found in these hogs was slightly smaller than the pathogen prevalence in the hogs that were skinned. This suggests that the aspirated material is not necessarily the primary cause for the biological contamination that is found in the hog lungs. Table 3 shows the pathogens prevalent in the lung tissue under the two different processing methods that were used on the hogs (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. The mold counts for all the samples were <10 CFU/g, meaning there was very little, if any, prevalence of mold in the lungs. The yeast counts for the lungs from the sows were all <10 CFU/g, however, higher yeast counts were found in the young market hogs. The average *Staphylococcus aureus* counts for both the young market hogs and the sows were <10 CFU/g. The aerobic plate counts were high for both the sows and the 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 4).

## Appendix

**Table 1.** Pathogen prevalence in pooled samples<sup>1</sup>

Pathogen	Total samples, young <sup>2</sup>	Positives	% Positives	Total samples, mature <sup>3</sup>	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's	32	10	31.3%	16	5	31.3%	48	15	31.3%
Yersenia 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%

<sup>1</sup>Pooled sample contains lung tissue from 5 separate hogs.

<sup>2</sup>Market ready barrows and gilts.

<sup>3</sup>Sows which have farrowed at least one litter.

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**Table 2.** Number of STEC's found in pooled samples that tested positive

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	<u>Number of STEC's<sup>1</sup></u>					
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>
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

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<sup>1</sup>The total number of the six major STEC's found per pooled sample.

**Table 3.** Pathogens prevalent using different processing techniques

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

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**Table 4.** Mold, Yeast, Staphylococcus aureus, and Aerobic Plate Counts in pooled lung samples

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

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## Appendix – Microbiological Procedures

### STEC Procedure:

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#### 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<sup>2</sup> STEC suite followed by cultural isolation. The BAX<sup>2</sup> 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 *stx*1 and *stx*2. Two additional BAX<sup>2</sup> 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<sup>2</sup> 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  $\pm 0.1$  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<sup>®</sup> type bags (or equivalent) d. Incubators, static  $42 \pm 1^\circ\text{C}$  and  $35 \pm 2^\circ\text{C}$  e. PCR tube holder (Qualicon or equivalent). f. Cell lysis tube cooling block (Qualicon or equivalent) held at  $5 \pm 3^\circ\text{C}$  g. PCR cooling block (Qualicon or equivalent) held at  $5 \pm 3^\circ\text{C}$  h. Heating block set at  $37 \pm 2^\circ\text{C}$  i. Heating block set at  $95 \pm 3^\circ\text{C}$  j. Repeating pipettor to deliver  $200 \pm 20$   $\mu\text{l}$  and sterile tips k. Pipettor to deliver  $20 \pm 1$   $\mu\text{l}$ , and sterile disposable filtered tips l. Pipettor to deliver  $150 \pm 15$   $\mu\text{l}$ , and sterile disposable filtered tips m. Eight-channel pipettor to deliver  $30 \pm 3$   $\mu\text{l}$ , and sterile disposable tips n. Pipettor to deliver  $5 \pm 1$   $\mu\text{l}$ , 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<sup>®</sup> 8 strip tubes, ISC Bioexpress, T-3120-5 or equivalent) q. Pipettor or pipettes to deliver 5 ml r. Dupont Qualicon BAX<sup>®</sup> System Q7 Instrument s. BAX<sup>®</sup> System Real-time PCR Assay STEC Screening (Part # D14642964) held at  $5 \pm 3^\circ\text{C}$  t. BAX<sup>®</sup> System Real-time PCR Assay STEC Panel 1 (Part # D14642970) held at  $5 \pm 3^\circ\text{C}$  u. BAX<sup>®</sup> System Real-time PCR Assay STEC Panel 2 (Part # D14642987) held at  $5 \pm 3^\circ\text{C}$  v. Micropipettors for culture plating to deliver volumes ranging from 15-1000  $\mu\text{l}$  with sterile disposable filtered tips w. VITEK<sup>®</sup> 2 system x. GN cards for VITEK<sup>®</sup> 2 system (bioMerieux Vitek, Inc.) y. Heating block ( $95-99^\circ\text{C}$ ) 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  $\mu\text{m}$  Cell Strainer jj. MACS<sup>®</sup> Large Cell Separation Columns (Miltenyi Biotec # 422-02) kk. OctoMACS<sup>®</sup> Separation Magnet (Miltenyi Biotec # 421-09) ll. Multistand to support OctoMACS<sup>®</sup> Separation Magnet (Miltenyi Biotec # 423-03) mm. Tray, autoclavable, approximately 130 mm x 83 mm for use with the OctoMACS<sup>®</sup> 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  $\pm 2\%$ . 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<sup>®</sup> Real-time PCR Controls

a. stx/eah 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/eah presumptive PCR / stx/eah 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 10<sup>9</sup> 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 10<sup>8</sup> 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<sup>®</sup> 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<sup>®</sup> 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  $200 \pm 20$   $\mu$ l lysis reagent to each cell lysis tube.
- Heat the filled lysis tubes for  $20 \pm 1$  minute at  $37 \pm 2^\circ\text{C}$ . Aseptically transfer 1 ml of the poultry mix enrichment sample to a sterile 1.5 ml microcentrifuge tube.
- Centrifuge at a setting of  $1,500 \times g$  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  $10,000 \times g$  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  $\mu$ l of PCR grade water either by vortexing or using the pipet tip.
- Add 5  $\mu$ l of the suspension directly to the pre-heated lysis buffer that was prepared during the initial steps.
- Heat the inoculated lysis tubes for  $10 \pm 1$  minute at  $95 \pm 3^\circ\text{C}$ . 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  $2-8^\circ\text{C}$  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  $2-8^\circ\text{C}$  for up to 7 days or at  $-20 \pm 3^\circ\text{C}$  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  $35 \pm 2^\circ\text{C}$ .

#### 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<sup>2</sup> real-time PCR and biochemical identification. The confirmatory BAX<sup>2</sup> 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<sup>2</sup>, 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  $\mu\text{l}$  of Molecular Grade Water (for up to 5 colonies). b. Add 5  $\mu\text{l}$  from the suspension to a BAX<sup>2</sup> lysis tube. Heat lysate at  $37 \pm 2^\circ\text{C}$  for  $20 \pm 1$  minute then  $95 \pm 3^\circ\text{C}$  for  $10 \pm 1$  minute then cool for 5-30 minutes in cooling block. Add 30  $\mu\text{l}$  of the lysate to the BAX<sup>2</sup> 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  $35 \pm 2^\circ\text{C}$  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  $35 \pm 2^\circ\text{C}$  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 $\square$  real-time PCR, prepare a template by suspending an agglutination positive colony from the SBA plate in 50  $\mu\text{l}$  of Molecular Grade Water and adding 5  $\mu\text{l}$  of this suspension to BAX $\square$  lysis buffer. e. Continue with the BAX $\square$  system protocol from the "Perform Lysis" step. The BAX $\square$  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 $\text{\textcircled{R}}$  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 $\square$  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 $\square$  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  $35 \pm 2^\circ\text{C}$ , and then store them at  $2-8^\circ\text{C}$ . 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 $\text{\textsuperscript{TM}}$  or lyophilize.

5B.10 Selected References Arthur TM, Barkocy-Gallagher GA, Rivera-Betancourt M, Koohmaraie M (2002) Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* on carcasses in commercial beef cattle processing plants. *Appl Environ Microbiol.* 68(10): 4847-52. Bhagwat, A.A, L., Chan, R Han, J Tan, M. Kothary, J. Jean-Gilles and B. D. Tall. (2005) Characterization of Enterohemorrhagic *Escherichia coli* Strains Based on Acid resistance phenotypes. *Infect Immun* 73(8): 4993-5003. Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA. (2005) Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. *J Infect Dis.* 192(8): 1422-9. Grant, MA (2004) Improved Laboratory Enrichment for Enterohemorrhagic *Escherichia coli* by Exposure to Extremely Acidic Conditions. *Appl Environ Microbiol* 70(2): 1226-1230. Possé B, De Zutter L, Heyndrickx M, Herman L. (2008) Novel differential and confirmation plating media for Shiga toxin-producing *Escherichia coli* serotypes O26, O103, O111, O145 and sorbitol-positive and -negative O157. *FEMS Microbiol Lett.* 282(1): 124-31. Richmond, J.Y. and R.W. McKinney (ed.). 2007. Biosafety in Microbiological and Biomedical Laboratories, 5 th ed. U.S. Government Printing

Office, Washington, D.C. Tillman GE, Wasilenko JL, Simmons M, Lauze TA, Minicozzi J, Oakley BB, Narang N, Fratamico P and WC Cray Jr (2012) Isolation of Shiga toxin-producing Escherichia coli serogroups O26, O45, O103, O111, O121, and O145 from ground beef using modified rainbow agar and post-immunomagnetic separation acid treatment. J Food Prot. Sep;75(9):1548-54. Wasilenko JL, Fratamico PM, Narang N, Tillman GE, Ladely S, Simmons M and WC Cray Jr (2012) Influence of primer sequences and DNA extraction method on detection of nonO157 Shiga toxin-producing Escherichia coli in ground beef by real-time PCR targeting the eae, stx, and serogroup-specific genes. J Food Prot. Nov;75(11):1939-50.

## **Strep 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](http://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](http://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

### **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.

3Petrifilm™ Aerobic Count Plates Reminders for Use Petrifilm Petrifilm Storage Sample Preparation Inoculation 1 2 3 4 5 6 7 8 9 Continued - over 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 KH<sub>2</sub>PO<sub>4</sub> 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 HCl.

### **Campylobacter Procedure**

#### **BAX® System Protocol**

1. Create rack file and warm up cyclor.
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 cyclor and run program.
7. Review results.

### **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 pre-labeled 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.

## Salmonella Procedures

[http://www.biomerieux-usa.com/sites/subsidiary\\_us/files/doc/vidas\\_easy\\_slm\\_flyer\\_8-19f-1.pdf](http://www.biomerieux-usa.com/sites/subsidiary_us/files/doc/vidas_easy_slm_flyer_8-19f-1.pdf)

## Staphylococcus aureus Procedures

### 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. Tolidine 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.

## Yersenia Procedures

A.

Equipment and materials

1. Incubators, maintained at  $10 \pm 1^\circ\text{C}$ ,  $\pm 35\text{-}37^\circ\text{C}$
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 accommodate 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. Cefsulodin-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.  $\beta$ -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**

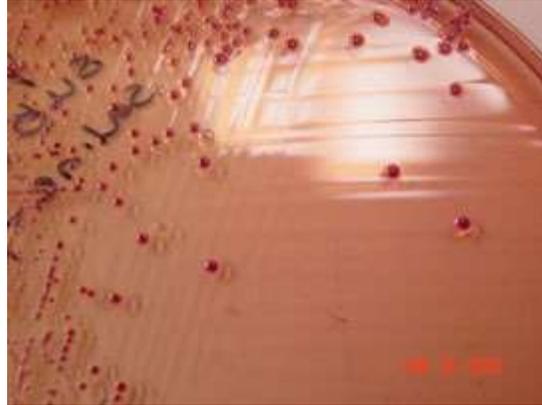
- › **Lactose negative colonies**
- › **flat, colorless, or pale pink**
- › **1-2 mm diameter**



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<sub>2</sub>S (KA-- ) reaction in LAIA, which are also urease-positive, are presumptive *Yersinia*. Discard cultures that produce H<sub>2</sub>S 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 positive)
- › *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).

0. Decarboxylase basal medium (Falkow) (M44), supplemented with each of 0.5% lysine, arginine, or ornithine; overlay with sterile mineral oil
1. Phenylalanine deaminase agar (M123)
2. 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)**



3. Tryptone broth ([M164](#))
4. Indole test (see instructions at end of chapter)
5. 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)
6. 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
7. Simmons citrate agar ([M138](#))
8. Veal infusion broth ([M173](#))
9. 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,  $\beta$ -D-Glucosidase, and pyrazinamidase (Table 2).
10. Pyrazinamidase agar slants (48 h) (see instructions at end of chapter)
11.  $\beta$ -D-glucosidase test (30°C, 24 h) (see instructions at end of chapter)
12. 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.

#### G. 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.

#### H. Pathogenicity testing

0. **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)**



1. **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.
2. **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.**



3.

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).

I. 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.

J. *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).

0. **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.

1. **Isolation and identification.** Continue as in E-H, above, noting biochemical differences (Table 1). Notably, *Y. pseudotuberculosis* strains are ornithine-, sorbitol-, and sucrose-negative.

Table 1. Biochemical Characteristics<sup>(a)</sup> of *Yersinia* species (2, 9, 10, 52)

Reaction	<i>Yersinia</i> species										
	<i>Y. pestis</i>	<i>Y. pseudotuberculosis</i>	<i>Y. enterocolitica</i>	<i>Y. intermedia</i>	<i>Y. frederiksenii</i>	<i>Y. kristensenii</i>	<i>Y. aldovae</i>	<i>Y. rohdei</i>	<i>Y. mollaretii</i>	<i>Y. bercovieri</i>	<i>Y. ruckeri</i>
Lysine	-	-	-	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	+(c)	+	+	+	+	+	+	+	+
Motility at RT (22-26°C)	-	+	+	+	+	+	+	+	+	+	+
35-37°C	-	-	-	-	-	-	-	-	-	-	-
Urea	-	+	+	+	+	+	+	+	+	+	-
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+/-	-	+	+	+	+	+	+	+	+	-
Cellobiose	-	-	+	+	+	+	-	+	+	+	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	+/-(+)	+/-(+)	+/-(+)	+/-(+)	+	-	+/-	-	-
Sucrose	-	-	+(c)	+	+	-	-	+	+	+	-
Rhamnose	-	+	-	+	+	-	+	-	-	-	-
Raffinose	-	+/-	-	+	-	-	-	+/-	-	-	-
Melibiose	-	+/-	-	+	-	-	-	+/-	-	-	-
Simmons citrate	-	-	-	+/-	+/-	-	-	+	-	-	+
Voges-Proskauer	-	-	+/-(+)	+	+	-	+	-	-	-	-
Indole	-	-	+/-	+	+	+/-	-	-	-	-	-
Salicin	+/-	+/-	+/-	+	+	-(+/-)	-	-	+/-	(+)	-
Esculin	+	+	+/-	+	+	-	+	-	(+)	(+)/-	-
Lipase	-	-	+/-	+/-	+/-	+/-	+/-	-	-	-	-
Pyrazinamidase	-	-	+/-	+	+	+	+	+	+	+	-

<sup>a</sup>+ = positive after 3 days at RT, (+) = positive after 7 days at RT.

<sup>b</sup> Some strains of *Y. intermedia* are negative for either Simmons citrate, rhamnose, and melibiose, or raffinose and Simmons citrate.

<sup>c</sup> Some biotype 5 strains are negative.

Table 2. Biotype scheme <sup>(a)</sup> for <i>Y. enterocolitica</i>							
Biochemical test	Reaction for biotypes <sup>(b)</sup>						
	1A	1B	2	3	4	5	6
Lipase	+	+	-	-	-	-	-
Esculin/salicin (24 h)	+/-	-	-	-	-	-	-
Indole	+	+	(+)	-	-	-	-
Xylose	+	+	+	+	-	V	+
Trehalose	+	+	+	+	+	-	+
Pyrazinamidase	+	-	-	-	-	-	+
β -D-Glucosidase	+	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+/- <sup>(c)</sup>	+	(+)	-

<sup>a</sup> Based on Wauters (51).

<sup>b</sup> ( ) = Delayed reaction; V = variable reactions.

<sup>c</sup> 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.

## Pyrazinamidase 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  $\text{NaH}_2\text{PO}_4$  (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.