United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health and Science
Laboratory QA/QC Division
950 College Station Road
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Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: PLG 0001.01

Effective Date: 1/30/04

Description and purpose of change(s):

The use of this modified histologic procedure for the detection of central nervous system tissue has been extended to include porcine in addition to bovine tissue.

This method has also been extended to include the identification of bovine dorsal root ganglia (DRG)/sensory ganglia as violative material.

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Title: Detection of Central Nervous System Tissue and Dorsal Root Ganglia in Beef and Central Nervous System Tissue in Pork Comminuted Meat Products by Histologic Examination of Hematoxylin and Eosin Stained Slides and Glial Fibrillary Acidic Protein Immunohistochemistry		
Revision: **01**	Replaces: Original	Effective: 1/30/04

Procedure Outline

1.1 Introduction

1.1.1 General

This procedure describes the use of histologic examination of Hematoxylin and Eosin (H&E) stained tissue slides and Glial Fibrillary Acidic Protein (GFAP) immunohistochemistry to identify central nervous system (CNS) and dorsal root ganglia/sensory ganglia (DRG/SG) tissue in beef and to identify CNS tissue in pork comminuted meat products. CNS tissue includes both brain and spinal cord. DRG/SG include dorsal root ganglia, trigeminal ganglia, and other sensory ganglia – many of which are located in or near the head and are often associated with the cranial nerves. Of these ganglia only the DRG and trigeminal ganglia are considered violative tissues by FSIS. Morphologically it is impossible to differentiate the source of fragments of sensory ganglia. If the bones or tissues used to produce the product are known (for example only vertebra used) the origin of ganglia may be assumed (in the case of the example the sensory ganglia

would be assumed to be DRG). If however the bones or tissues used to produce the product are mixed in nature or associated with the skull, then the sensory fragment can only be identified as being sensory ganglia. For regulatory purposes if fragments of sensory ganglia are identified they will be designated as DRG/SG and coded as violative.

This procedure is used by the staff of the FSIS Eastern Laboratory pathology section for testing both regulatory and baseline study samples. It is a modification of an earlier procedure, which uses H&E stained tissue slides and immunohistochemistry for neurofilament, GFAP and synaptophysin to identify CNS tissue in comminuted meat.

The original method may still be used at the discretion of the staff pathologist. However, the modified method has been shown in a blind retrospective study to be as effective as the original method for the detection of CNS tissue in comminuted meat products and DRG/SG in beef. All samples identified as positive for CNS or DRG/SG tissues are subject to confirmation by independent histological examination by a second FSIS pathologist.

1.1.2 Limits of Detection

This procedure, as evaluated by a controlled study examining comminuted product spiked with seven different levels of bovine or porcine spinal cord, was found to have a sensitivity of detecting CNS tissue at least to the 0.25% level. A similar study examined comminuted product spiked with six different levels of bovine DRG and found to have a sensitivity of detecting DRG at least to the 0.5% level.

1.2 Safety Precautions

No additional safety precautions other than good laboratory practices for histological techniques are necessary while processing comminuted meat samples for histologic examination.

1.3 Quality Control Procedures

1.3.1 Control Tissues

a. Control blocks shall contain spinal cord, DRG and peripheral nerve of the appropriate species.

b. Control tissues should have been formalin-fixed for no more than 48 hours, routinely processed for histology, and then paraffin-embedded.

c. The immunoreactivity of sections for GFAP should have been previously verified by immunostaining and microscopic examination.

1.3.2 Control Slides

a. At least one set of control slides (one control positive for GFAP and one control negative for GFAP) will be run for each batch of slides prepared on the DAKO Autostainer.

b. Two sections from the control block are sectioned with a microtome at 4-6 microns, floated on a warm water bath, and placed on silanized or positively charged glass microscope slides.

c. Sections are dried by heating in an oven for 30 minutes to overnight.

d. Paraffin is removed by repetitive sequential immersion in xylene. Slides are rehydrated in 100% ethanol and 95% ethanol (three changes, 5-10 minutes each of xylene, 100% ethanol, and 95% ethanol) and rinsed in distilled water.

e. Antigen retrieval is performed with Citra buffer (follow manufacturer's instructions) and sections are then rinsed well with buffer.

f. For the positive control, the diluted antibody to GFAP is applied to the section and incubated at room temperature for 20-40 minutes (preferred time 30 minutes). Recommended incubation time and temperature are supplied by manufacturer of antibody, or determined by previous serial dilutions. For the negative control the primary antibody is omitted or a spurious monoclonal antibody is used.

g. Tissue sections are rinsed well with buffer, the secondary antibody (link) is applied and is incubated for 10-30 minutes at room temperature (preferred time 20 minutes for beef; 10 minutes for pork). Sections are rinsed well with buffer and then the label is applied. Sections are incubated for 10-30 minutes at room temperature (preferred time 20 minutes) and rinsed with buffer.

h. The substrate is applied (if using Fast Red substrate from Biogenex Laboratories, add one fast red tablet to a bottle of napthol phosphate buffer and shake until dissolved, let sit approximately one hour before using) and incubated for 1-8 minutes at room temperature (preferred time 2 minutes for beef; 3 minutes for pork). Color development of the positive control may be watched under the microscope. No color development should take place on the negative control.

i. Sections are rinsed well with distilled water or buffer and counterstained with Gill's hematoxylin 10-30 seconds (preferred time 15 seconds). Sections are then rinsed well with 1% acetic acid water and then tap water. Sections are then blued in Scott's tap water for 15-60 seconds and rinsed well with tap water.

j. Slides are drained and placed on a horizontal surface. Two to five drops of Crystal Mount are applied to the tissue sections. Slides are rotated so the entire section is covered, and drained individually to remove any excess or bubbles.

k. Slides are placed horizontally in a warm oven for 10 to 30 minutes or until dry and then removed and allowed to cool. Slides are quickly individually dipped into xylene and coverslipped using permanent mounting media.

1.4 Equipment, Supplies, and Reagents

1.4.1 Equipment

a. Sakura Tissue-Tek® DRS 601 Automatic Slide Stainer or equivalent
b. Autostainer Dako Model LV-1 Automatic Slide Stainer or equivalent
c. Tissue Processor
d. Embedding Center
e. Microtome
f. Water Bath
g. Microscope

1.4.2 Supplies

a. Microscope Cover Glass
b. Microscope Slides

c. Charged Microscope Slides
d. Paraffin Paraplast+ or equivalent
e. Biopsy bags
f. Processing Cassettes
g. Mounting Media
h. Neutral buffered formalin solution, 10% formalin

1.4.3 Reagents

a. Glial Fibrillary Acidic Protein (Monoclonal) – Predilute (Diluted 1:10 to 1:20) Biogenex Laboratories Catalog #AM020-5M (for beef). Glial Fibrillary Acidic Protein [GF-01](Monocolonal) – Predilute (Diluted 1:400 – 1:800), GeneTex, Inc. Catalog # ab7806 (for pork)
b. Antigen Retrieval-Citra 10X Biogenex Laboratories Catalog #HK086-9K, or equivalent
c. Super Sensitive Multilink, Alkaline Phosphatase Detection Kit Laboratories Catalog #LA000-UL, or equivalent (for beef). Biotinylated Secondary antibody (link) Goat Anti-Mouse IgG-Rabbit and Swine Adsorbed, Biocare Medical Catalog # GM612H, or equivalent (for pork). Use the alkaline Phosphatase-Labeled streptavidin (from the Super Sensitive Immunodetection System listed.
d. Fast Red Substrate Biogenex Laboratories Catalog #HK182-5K, or equivalent
e. Tris Buffered Saline Automation Buffer with Tween 20
f. Gill's Hematoxylin
g. Scott's Tap Water
h. Crystal Mount Aqueous/Dry Mounting Media Biomeda Corporation Catalog #M03, or equivalent
i. Eosin
j. 1% Acetic Acid Water

1.5 Preparation of Samples

1.5.1 Preparation of Blocks and Slides from Comminuted Meat

a. Four quantities of the comminuted meat product that are sufficient to create four blocks of tissue approximately 25mm x 20 mm x 5 mm are removed from four different sites in the sample. These tissues are placed in four separate

individually identified plastic tissue cassettes and each cassette is placed in biopsy filter bag. The lid is attached to secure the tissue in the biopsy bag and immersed in 10% neutral buffered formalin solution.

b. Tissue blocks are fixed for several hours to overnight. Then the blocks are removed from the formalin solution and routinely processed for histology. After processing, tissue blocks are embedded in paraffin wax.

c. Sections from each block are cut with a microtome (each section should be 4-6 microns in thickness) and sections are floated on a warm water bath. Selected sections are placed on plain glass (for hematoxylin/eosin [H&E] stained sections) or silanized or positively charged glass microscope slides (for immunohistochemical sections). Two quality sections from each block are needed, one for H&E sections and one for immunohistochemical sections.

d. Sections are dried by heating in an oven for 30 minutes to overnight.

e. Paraffin is removed by repetitive sequential immersions in xylene. Slides are rehydrated in 100% ethanol, and 95% ethanol (Two to three changes, 5-10 minutes each of xylene, 100% ethanol, and 95% ethanol). Sections are then rinsed in distilled water.

1.6 Hematoxylin and Eosin Staining

The four sections placed on plain glass microscope slides (one section from each block) are used for creating hematoxylin and eosin (H&E) slides. After deparaffinization and rehydration, the sections on the slides (hereafter referred to as sections) are:

a. Immersed in Hematoxylin solution for 1 minute. Then sections are rinsed in 1% acetic acid water and rinsed in tap water.

b. Sections are then rinsed in Scott's Tap Water, and rinsed in tap water.

c. Sections are then rinsed in 95% ethanol and immersed in Eosin solution for 30 seconds.

~~d.~~ Then sections are rinsed in 95% ethanol for 30 seconds and subsequently rinsed in several changes of 100% ethanol (approximately 30 seconds each rinse).

~~e.~~ Sections are cleared in several changes of xylene and are covered with a glass coverslip using synthetic mounting media.

1.7 Glial Fibrillary Acidic Protein Immunohistochemical Staining

a. Four sections (one from each block) are used for immunohistochemistry. After deparaffinization and rehydration, antigen retrieval is performed with Citra buffer (follow manufacturer's instructions) and sections are then rinsed well with buffer.

b. The diluted antibody to GFAP is applied to the sections and incubated at room temperature for 20-40 minutes (preferred time 30 minutes). Recommended incubation times and temperatures are supplied by manufacturer of antibody, or are determined by previous serial dilutions.

c. Tissue sections are rinsed well with buffer and the secondary antibody (link) is applied and incubated for 10-30 minutes at room temperature (preferred time 20 minutes for beef; 10 minutes for pork). Sections are rinsed well with buffer and the label is applied. Sections are then incubated for 10-30 minutes at room temperature (preferred time 20 minutes) and rinsed with buffer.

d. The substrate is applied (if using Fast Red substrate from Biogenex Laboratories, add one fast red tablet to a bottle of napthol phosphate buffer and shake until dissolved, let sit approximately one hour before using) and incubated for 1-8 minutes at room temperature (preferred time 2 minutes for beef; 3 minutes for pork). Color development may be watched under the microscope.

e. Sections are rinsed well with distilled water or buffer and counterstained with Gill's hematoxylin 10-30 seconds (preferred time 15 seconds), rinsed well with 1% acetic acid water, and then rinsed with tap water. Sections are then blued in Scott's tap water for 15-60 seconds and rinsed well with tap water.

f. Slides are drained and placed on a horizontal surface. Two to five drops of Crystal Mount are applied to the tissue sections. Slides are rotated so the entire section is covered, and then slides are drained individually to remove any excess or bubbles.

g. Slides are placed horizontally in a warm oven for 10 to 30 minutes or until dry and then removed and allowed to cool. Slides are quickly, individually dipped into xylene and a glass coverslip is attached using permanent mounting media.

1.8 Detection and confirmation procedures

1.8.1 Detection

All tissues on the eight microscopic slides are examined microscopically at various magnifications (at least 100x for immunostained slides). Samples are considered positive for CNS tissue if there is deposition of red chromagen on GFAP immunostained slides that is compatible with the deposition present on positive controls for CNS tissue. H&E stained slides are also examined in order to view tissue morphology and to eliminate fragmented peripheral nerve.

Samples are considered positive for DRG/SG if morphology of tissue fragments in the samples is consistent for peripheral ganglia containing sensory neurons and there is deposition of red chromagen on GFAP immunostained slides that is compatible with the deposition present on the positive control for DRG/SG.

1.8.2 Confirmation

Control slides for immunohistochemistry are examined to assure that there is no deposition of chromagen on the negative control slides and that the chromagen appropriately labels astrocyte processes on positive control slides. Only minimal staining of peripheral nerve should be present. Satellite cells of dorsal nerve root ganglionic neurons also stain with GFAP. Slides considered positive by microscopic evaluation for either CNS tissue or DRG/SG will be reviewed by a second analyst to verify findings.

1.9 Selected References

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