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ABSTRACT

This study investigated the usefulness of immunohistochemistry, histochemistry, and polarization microscopy in documenting the presence of spinal cord in ground product produced by advanced meat recovery systems (AMRS). A battery of diagnostic stains proved most helpful for definitive documentation of spinal cord in the AMRS product. Antibodies to neurofilament and glial fibrillary acidic proteins were useful for highlighting the presence of neural tissue in comminuted product but when used alone did not definitively differentiate central nervous system tissue from peripheral nerve. Polarscopy, trichrome staining, and immunostaining for synaptophysin were very useful for differentiating spinal cord from peripheral nerve. Spinal cord was found in 2 of 196 AMRS samples when only hematoxylin and eosin staining was used. In a later immunohistochemical subset of the survey study, spinal cord was found in 7 of 17 AMRS samples. More recently, spinal cord tissue has been found in 3 of 26 regulatory samples using hematoxylin and eosin stains combined with immunohistochemical techniques.

Mechanical systems have been developed that use pressure to detach meat from the bone without grinding of the bone. These advanced meat recovery systems (AMRS) are being used by a steadily increasing number of meat processors to recover meat tissue that normally adheres to the bones. These machines are used to improve the efficiency and yield of separating meat from the bone and also reduce the risk of repetitive motion to employees using a deboning knife.

The Food Safety and Inspection Service (FSIS) published a final rule on December 6, 1994, entitled “Meat Produced by Advanced Meat/Bone Separation Machinery and Meat Recovery Systems” (2, 3). The agency subsequently issued FSIS directive 7160.1 entitled “Meat Produced by Advanced Meat/Bone Separation Machinery and Meat Recovery Systems” on September 13, 1996, to provide guidance to field personnel inspecting AMRS operations (5). The 1994 final rule amended the definition of meat (9 CFR 301.2 rr) to include as “meat” product that resulted from advanced meat and bone separation machinery that does not crush, grind, or pulverize bones to remove attached edible skeletal muscle if the bones that emerge are comparable to those resulting from hand deboning. An upper calcium limit of 0.15% was also set for the product. AMRS product meeting these criteria is then allowed to be labeled as meat rather than as mechanically separated species.

Beef AMRS product has been labeled as finely ground beef and is often mixed with ground beef and used in restaurants, sold as ground beef in retail and wholesale grocery stores, and widely used in pizza toppings, tacos, and other foods that use ground beef. Pork AMRS products are labeled as finely ground pork or ground pork trimmings and are used primarily in sausages.

Consumer groups raised concerns that regulatory requirements for production of the “meat” product were not being met. They claimed that bones emerging from some of the AMRS equipment were crushed or pulverized. They thought, therefore, that the product could contain higher-than-acceptable levels of calcium and other bone components such as bone marrow.

In response to these concerns, a chemical and historical survey was conducted by the Office of Public Health and Science, FSIS, to provide analytical data about product obtained from beef neck bones processed by AMRS machinery and to compare product characteristics of hand-deboned meat and AMRS machine-deboned product. When spinal cord was detected in some of the AMRS product, a subset of the survey samples was selected for testing by immunohistochemical analysis to determine if additional suspect material was central nervous system tissue. It was important to differentiate peripheral nervous tissue from spinal cord, since peripheral nerve is normally found in small amounts in meat and is not considered to be a violation, but the presence of central nervous system tissue, such as spinal cord, in product labeled “ground beef” could be construed as product mislabeling. The presence of central nervous system tissue also raises concerns about disease-causing agents found more commonly in central nervous system tissue rather than in skeletal muscle.

The FSIS subsequently supplemented its instructions to inspection program personnel (directive 7160.2 entitled “Meat Prepared Using Advanced Meat Recovery Sys-
items”) on April 14, 1997, to clarify that AMRS product containing spinal cord is not meat. Routine regulatory samples are currently being submitted by inspection personnel, and the results of examinations of 26 of these samples are also included in this report. The purpose of the present study was to evaluate by light microscopy the combination of methods necessary to identify spinal cord in a comminuted meat product derived from the residual meat on beef neck bones (AMRS product) and to report the identification of central nervous system tissue in AMRS product.

MATERIALS AND METHODS

Selection of samples for immunohistochemical analysis. Survey participants were randomly selected from meat processing establishments that primarily used beef neck bones (vertebrae) in the AMRS equipment. Seven establishments using AMRS equipment and four establishments using hand deboning were selected to participate in the study. The AMRS establishments selected used one Protecon Trimline 60 Separator and six Hydra Separator machines. AMRS samples evaluated included 19 samples of product that had been subjected to the AMRS without desinewing and 177 samples that had been subjected to the AMRS equipment with desinewing, yielding a total of 196 AMRS samples. Sixty-four control samples were collected from four randomly selected establishments that hand deboned beef neck bones. Samples were collected at randomly selected times, 3 times a day, 3 days a week, for 3 weeks in each of the selected establishments. Ten grams of AMRS product or hand-deboned product filled two plastic histology cassette containers that were immediately placed into 10% neutral-buffered formalin before being shipped to the Pathology Section of the FSIS Eastern Laboratory, Athens, Ga. Two paraaffin-embedded blocks were prepared from each cassette, yielding four blocks to be evaluated from each original sample.

Histologic sections of hand-deboned meat and AMRS product were initially evaluated by light microscopy using hematoxylin and eosin (HE) staining techniques. As part of a subsequent phase of the survey, immunohistochemical assays were performed on a subset of these samples. Twenty-two samples were selected for immunostaining, including 15 AMRS product samples and seven control boneless beef samples, with each of the 11 establishments being represented at least once in this subset of samples. Two additional AMRS product samples, shown to contain spinal cord by HE staining, were specifically selected to immunohistochemically confirm the presence of spinal cord. The 26 regulatory samples were submitted after completion of the survey. These samples were AMRS product that was removed after the desinewing, many of the post desinewer AMRS product through fine screens to remove spinal cord was detectable by HE staining in any of the 177 samples. The power setting of the microwave was then reduced to 400 W and adjusted so that the oven cycled on and off every 20 to 30 s, allowing the solution to boil every 5 to 10 s during each cycle, for 6 more min (7, 12). Slides were cooled for 20 min and rinsed in several distilled water washes. After four subsequent washes in phosphate-buffered saline (PBS), sections were incubated with monoclonal antibodies to neurofilament (clone 2F11), synaptophysin (clone SY 38), or glial fibrillary acidic protein (GFAP) (clone G-A-5) for 30 min at room temperature. All primary antibodies were obtained from BioGenex for use with the supersensitive kits. After washing with PBS, sections were incubated with biotinylated anti-mouse immunoglobulins for 20 min at room temperature. Sections were washed four times with PBS and covered with the streptavidin-labeled alkaline phosphatase for 20 min (1, 15). Sections were washed four times with PBS, and the fast red substrate (BioGenex) was applied for 2 to 6 min. Sections were rinsed with deionized water, counterstained with Mayer’s hematoxylin for 15 s, rinsed with tap water, and treated with Scott’s tap water. Sections were covered with Crystal Mount (Biomedia Corporation, Foster City, Calif.) and dried, and coverslips were mounted with a permanent mounting media. Slides containing mounted sections were examined by light microscopy for the extent and pattern of chromogen deposition. Negative control incubations using murine ascites fluid (mouse supersensitive negative control) as the first antibody resulted in the absence of specific staining in the control tissues.

RESULTS

With examination of HE-stained slides alone, only 2 of 196 AMRS samples were found to contain spinal cord. In these 2 cases, which belonged to the group of 19 samples subjected to the AMRS machinery but not pressed through the desinewer, large sections of spinal cord were available for evaluation. Characteristic features unique to spinal cord that could be documented included a central canal lined by ependymal cells and dorsal and ventral horns of gray matter surrounded by white matter (Fig. 1A and 1B). The desinewer presses the product through fine screens to remove large pieces of connective tissue and in doing so breaks the product into 2- to 3-mm fragments. No anatomically intact spinal cord was detectable by HE staining in any of the 177 samples that were subjected to the AMRS machinery followed by the desinewer. Many of the post desinewer AMRS samples contained tissues compatible with central nervous system tissue, but the tissue was so fragmented and auto-lyzed that the larger structural features specific to spinal cord were not easily identified by routine histologic examination. Therefore, a subset of the post desinewer AMRS samples was selected for immunohistochemical evaluation to determine if this method could distinguish between neurologic tissue of spinal cord or peripheral origin in these smaller fragments of tissue.

Trichrome staining and microscopic polarization (10) of HE sections of spinal cord controls demonstrated colla-
gen within the dura mater surrounding the entire cord and following small blood vessels into the cord. Trichrome staining and polarization of HE sections of peripheral nerve demonstrated the presence of collagen surrounding each individual myelin sheath (Fig. 1C). No collagen surrounded myelin sheaths in the spinal cord (Fig. 1D).

Immunohistochemical reactivity of control spinal cord and peripheral nerve were as follows. Neurofilament staining of the spinal cord white matter was limited primarily to the axons (Fig. 2A), with faint staining surrounding neuroglial cell nuclei. Within gray matter of the spinal cord, immunoreactivity to neurofilament was present diffusely in the neuropil and was concentrated within the nonmyelinated axons. Scattered astrocytes, neurons, and ependymal cells were also immunopositive for neurofilament. Immunoreactivity to neurofilament was present only in axons of peripheral nerve (Fig. 2B).

The myelin sheaths and cytoplasm of neuroglial cells were brightly immunopositive for GFAP in the white matter of the spinal cord (Fig. 2C). In spinal cord gray matter, astrocytes and glial filaments scattered throughout the neuropil were immunopositive for GFAP. Schwann cells forming neurolemmal sheaths in peripheral nerves were variably positive for GFAP; these cells always stained much more faintly than glial cells in the spinal cord. Staining was too faint to capture with black-and-white photography.

Synaptic vesicles immunopositive for synaptophysin were most prominent in the gray matter neuropil (Fig. 2D), with scattered extensions into adjacent white matter. Nerve cell bodies were variably positive for synaptophysin. Sec-
Immunostains for neurofilament highlight axons in both spinal cord (A) and peripheral nerve (B), whereas antibodies to GFAP (C) stain the myelin sheaths of axons in spinal cord white matter most intensely. Antibodies to synaptophysin (D) react only with spinal cord gray matter, leaving spinal cord white matter (W) unstained. Bars = 100 μm.

...ations of peripheral nerve were uniformly negative for synaptophysin.

In the two AMRS samples determined to contain spinal cord by HE staining, immunohistochemical staining, triochrome staining, and polarization of the product confirmed staining patterns identical to the control spinal cord. Of the 15 AMRS samples subjected to the desinewer and also selected to be studied by immunohistochemical methods, spinal cord was detected in five of the samples (Fig. 3) and an additional two samples contained dorsal or ventral nerve root ganglia. Most samples contained small amounts of peripheral nerve. Immunohistochemical staining, triochrome staining, and polarization of the hand-deboned samples did not demonstrate the presence of spinal cord or nerve root ganglia. Only peripheral nerve was shown to be present in small amounts in the hand-deboned meat. In the AMRS samples that contained spinal cord, numerous 1 × 3 to 5-μm filaments positive for neurofilament and GFAP were also present. These filaments were not as common in AMRS samples not found to contain spinal cord and were not present in the hand-deboned samples.

Twenty-six regulatory samples have been analyzed thus far. Two bovine samples and one porcine sample were found to contain spinal cord.

**DISCUSSION**

The identification of spinal cord tissue by HE staining in AMRS samples not subjected to desinewing suggested that this tissue would also be present in the AMRS product subjected to desinewing. Standard light microscopy was not adequate for detection of small autolytic fragments of spinal cord tissue and did not allow for discrimination between fragments of spinal cord or peripheral nerve (a normal component of meat). Immunohistochemical methods, which
FIGURE 3. In HE-stained sections of AMRS product (A), suspect material that contains swollen axons (arrows) is identified as central nervous system tissue after examination of replicate sections immunostained for neurofilament (B), GFAP (C), and synaptophysin (D), which identified numerous individual synaptic vesicles (arrows). Bars = 100 μm.

rely on the presence of reactive antigens in tissue sections, offered a promising alternative for detection of spinal cord in ground product. Three characteristic protein markers of neurologic tissue were selected for evaluation in our study. Two intermediate filament markers, neurofilament and GFAP (14), were selected, since intermediate filaments are more resistant to autolysis than other cellular proteins. Synaptophysin (6) was selected as a differential marker, since it is not present in peripheral nerve or skeletal muscle except in minute quantities at the neuromuscular junction.

Intermediate filaments are filamentous, developmentally regulated structures 10 nm in diameter that compose part of the cytoskeleton of nearly all mammalian cells. There are five different classes of intermediate filaments, and each class, with the exception of vimentin, is restricted to a limited number of cell types. GFAP, a 50- to 52-kDa molecule isolated more than 20 years ago, was the first polypeptide to be identified as the major component of an intermediate filament class. The distribution of GFAP is thought to be limited to a type of glial cell known as an astrocyte, making GFAP a central nervous system–specific protein. However, our report shows immunopositivity present in peripheral nerves, suggesting the presence of GFAP or a shared epitope within Schwann cells of the peripheral nervous system. Because of the faint GFAP immunoreactivity of some peripheral nerves in our study, the anti-GFAP antibody was not as definitive in differentiating spinal cord from peripheral nerve as was anticipated from a review of the literature. However, the contrast in staining intensity allows some differentiation, since the white matter of spinal cord stains very intensely with antibodies to GFAP, whereas peripheral nerve typically stains only faintly. Other factors, such as fixation and autolysis, can, however, affect staining intensity.

In an autolyzed material such as the AMRS product,
the architecture of the original tissue is so disrupted that discernment of spinal cord is difficult or impossible with the use of HE stains alone. Neurofilaments were surprisingly resistant to autolytic degradation, and the brightly positive reactions with antineurofilament antibody were, therefore, very useful for documenting the presence of neural tissue in the AMRS product. Neurofilament is present in equal amounts and in equal intensity of staining in spinal cord and peripheral nerve. Therefore, the presence of neurofilament by itself is not useful for differentiating spinal cord from peripheral nerve.

Synaptophysin is a 38-kDa transmembrane glycoprotein of presynaptic vesicles localized in the brain, spinal cord, retina, neuromuscular junctions, pancreatic islets, and adrenal medulla. Since peripheral nerve does not contain synaptophysin, synaptophysin immunopositivity was useful for discriminating spinal cord from peripheral nerve. However, spinal cord was only positive for synaptophysin when gray matter of the cord or white matter in near proximity to the gray matter was present in the sample. Many of the AMRS samples containing spinal cord were not positive for synaptophysin. This is not surprising, since only 10% of the spinal cord is gray matter.

Polariscopy is an inexpensive, quick method for the detection of collagen in tissues, since collagen exhibits anisotropic characteristics thought to be related to its protein structure (13). Anisotropic substances are those in which the velocity of transmitted white light varies with the direction of propagation of the light and in which the white light is resolved into groups of waves that vibrate in two mutually perpendicular planes. Anisotropic substances appear alternately bright and dark when rotated between crossed polarizing prisms. By means of the polarizing microscope, such substances can be readily distinguished from isotropic substances (10). The differential distribution of collagen fibers (detected by polariscopy and trichrome staining) in the spinal cord and peripheral nerves proved to be very useful in differentiating the two tissues.

Neurofilament (14), GFAP (14), and synaptophysin (6) are nervous system–specific proteins, and antibodies to these proteins have been most widely used to determine the cell of origin for poorly differentiated tumors. In this study, antibodies to these proteins were useful for documenting the presence of neural tissue. These immunohistochemical techniques, when combined with polariscopy and HE staining, were useful for differentiating peripheral nerve from central nervous system tissue in AMRS product. Synaptophysin, when present, was useful for documenting the presence of spinal cord gray matter. Our results demonstrate the value of immunochemical techniques in revealing the presence of spinal cord in a ground meat product.

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REFERENCES