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Original Article

Application of histochemical and immunohistochemical techniques for detection of lung tissue in cooked sausage

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Abstract

Background: Using unauthorized tissues in sausage is a common food adulteration in some parts of the world. **Aims:** This study was designed to compare the accuracy of histochemical and immunohistochemical techniques for the detection of lung tissue in cooked sausage samples. **Methods:** Samples with different levels of sheep lung tissues (1, 2.5, and 5%) and a control group were prepared and stained histochemically using H&E, Masson trichrome, and Periodic Acid-Schiff (PAS) stainings, and immunohistochemically using two different commercially-available antibodies of TTF1 Pan-cytokeratin. **Results:** The highest positive results of lung tissue detection were achieved in sausage samples stained with anti-TTF1 immunohistochemical staining method. Both anti-TTF1 and anti-pan-cytokeratin immunohistochemical techniques detected all contaminated sausage samples treated with 50 g/kg lung tissues. Anti-TTF1 staining method had the highest odds ratio (7.4), followed by anti-pan-cytokeratin method (6.0). Reversely, PAS staining method had the lowest odds ratio (0.21), followed by Masson trichrome method (1.7). Additionally, anti-TTF1 method had the highest (1.8-31.0) confidence intervale (95%), while PAS had the lowest (0.02-2.1). Totally, the odds ratio of lung tissue detected by immunohistochemical methods were higher than those detected by histochemical staining. **Conclusion:** This is the first report on the comparison of histochemical and immunohistochemical techniques for lung tissue detection in cooked sausage. Anti-TTF1 immunohistochemical staining proved to be the most useful technique for the detection of unauthorized lung tissue in cooked sausages.

Key words: Food adulteration, Immunohistochemistry, Lung tissue, Sausage

Introduction

Sausage is an acceptable ready-to-eat food product among all age groups, worldwide. The consumption of sausage has increased, and therefore consumers need assurance regarding the quality and safety of sausage products. In spite of many efforts of producers to improve the quality of sausage, the public's view has been partly negative about these ready-to-eat products due to recent issues raised in food adulteration and recent negligence about the presence of contaminated central nervous system (CNS) with bovine spongiform encephalopathy (BSE) in meat products (Chen *et al*., 2013; Pandey *et al*., 2020).

Different types of undesirable and sometimes detested ingredients among customers are added to meat products because of their low price or technological properties in different processes of sausage production (Ahmed *et al*., 2020). Strikingly, a worldwide increase in food prices especially for meat has caused some producers to use undesirable tissues such as the spleen, liver, mammary gland, and lung in meat products (Dehghan Shahreza, 2016). Since animal offal can be contaminated with bacteria, viruses, foreign bodies, and parasites or even can be served as a pathway for various types of environmental toxicants to enter the human body, the use of these resources in meat products should be monitored more carefully. Given this point for lung tissue, some studies suggested that the significant uptake of cadmium (Cd) and manganese (Mn) in dairy farms can happen through the lung (Sunderman, 2001; Bressler *et al*., 2004; Roggeman *et al*., 2014; ShahbaziGahrouei and Keshtkar, 2016). Also, the animal lung can be a potential vehicle for food-borne bacteria including listeria (Kuan *et al*., 2013). Thus, awareness about animal offal including lung can play an important role in food quality and safety control, especially due to the European Union's strict regulations on the use of specified risk material (SRM) (2000/418/EC), their agreement for labeling all the permitted edible parts (2001/101/EC) and more careful inspection of the used offal in meat products (Sultan *et al*., 2004).

The importance of efficient techniques for evaluating the composition of comminuted meat products has increased (Koolmees and Bijker, 1985; Koolmees *et al*., 1986; Meret *et al*., 1998; Hajmeer *et al*., 2003), for example, immunochemical and immunohistochemical methods have been used to detect CNS as unauthorized tissue in commercial beef sausages or homemade meat products (Boon, 1990; Lücker *et al*., 1999; Schmidt *et al*., 1999; Lücker *et al*., 2000). Regarding this point, there have been some commercial kits for the detection of CNS tissue in meat products (Yeşilbağ and Kalkan, 2005). However, no kit has been designed for the detection of other tissues. Hence, the present research was conducted to the comparison of the performance of histochemical and immunohistochemical techniques for the detection of the lung tissue as adulteration in cooked sausage.

Materials and Methods

Reagents

The anti-thyroid transcription factor 1 (TTF1) and Pan-cytokeratin antibodies were obtained commercially from Dakocytomation Company, Denmark.

Sausage production

The production procedure of a lyoner meat-type product was as follows: after mincing calf meat with a mincer machine with 3 mm plates of stainless steel (Pars Khazar Co., Iran), they were mixed with ice, salt, ascorbate and phosphate in a food maker (Moulinex Co., France) until the temperature reached 10°C. Then, Soya, flour, starch, and spices were added to the mixture and the final mixture was chopped until a suitable consistency was achieved at a temperature of 15°C. This mixture was considered as the control group (group A), then different amounts of minced lung tissue were added to the chopped product to form groups B, C, and D including 10, 25, and 50 g/kg lung tissue, respectively. They were mixed again, filled to the commercial artificial casing, and located in a water bath with a core temperature of 72°C for 2.5 h. Experimental sausage production was repeated 5 times.

Histochemical staining

From each sausage, one block (1 × 1 × 1 cm) was taken. A total of 20 blocks were sampled from 4 different groups. Samples were then fixed in a 10% neutral buffered formalin solution and processed according to the routine paraffin wax method. From each Paraffin block, 5 sections (5 µm) were cut. Three sections were stained histologically by hematoxylin and eosin (H&E), Masson trichrome, and Periodic Acid-Schiff (PAS). Totally, 100 sections were prepared and all fields of each slide were carefully examined with magnification of ×100 and ×400 by a light microscope.

Immunohistochemical staining

The tissue sections were stained immunohisto-chemically using an avidin-biotin peroxidase complex (ABC) method (Haines and Chelack, 1991). The sections were deparaffinized and rehydrated by sequential immersion of the slides in xylene, graded concentrations of ethanol and distilled water. Then slides were immersed in a citrate solution and heated in a microwave oven for 3 min until the solution started to boil. Endogenous tissue peroxidase activity was blocked by immersion of the slides in a solution of 0.5% H_2O_2 in methanol for 30 min at room temperature. Sections were treated with phosphate-buffered saline (PBS) supplemented with 4.0% normal goat serum to block non-specific background staining, then sections were incubated with monoclonal antibodies such as TTF1 and Pan-cytokeratin with a concentration of 1:100 in PBS for 60 min. After washing (3 times and 5 min for each time) with PBS/Tween, sections were incubated with a biotinylated goat anti-rabbit antibody for 30 min. They were washed with PBS/Tween, flooded with avidin-biotin complex peroxidase solution and again were washed 3 times with PBS/Tween. The peroxidase activity was visualized with 1 mg/ml DAB (3, 3'-Diamino-Benzidine) in PBS supplemented with H_2O_2 (10 µL of 50% H_2O_2 in 5 ml PBS) as a chromogen. After washing with PBS/Tween, sections were counterstained with Mayer's hematoxylin for 1 min. Finally, they were dehydrated, mounted and examined by light microscopy.

Statistical analysis

The ratio of positive-diagnosed slides to the total slides of each staining was considered for further statistical analysis. Then, the logistic regression model was used to calculate odds ratios (OR, multiplicative), corresponding 95% confidence intervals (CI) and significant differences between histochemical techniques. These methods were considered reference methods and immunohistochemical methods were compared with them.

Results

Analysis of staining methods

The current survey was done to evaluate the accuracy of histochemical and immunohistochemical techniques to detect unauthorized lung tissue in sausage samples. Sausage samples with different amounts of lung tissue were stained with three staining methods.

Figure 1 signifies the findings of the H&E staining method for the detection of lung tissues. Alveolar-like structure with thin septa was seen in the H&E staining method which was considered positive for lung tissue. In H&E sections of the control group, some homogenous eosinophilic materials and skeletal fibers were observed, but in the experimental groups, thin-walled and alveolar-like structures as cleft-like features were identified as well as skeletal fibers with degenerative changes (Fig. 1). Figure 2 signifies the findings of the Masson trichrome staining method for the detection of lung tissue in studied sausage samples. More clear alveolar-like structure and hyaline cartilage were found in sausage samples in Masson trichrome staining method which was considered

positive for lung tissue. In Masson trichrome staining, alveolar ducts were stained red to purple, and connective tissue between alveolar septa and hyaline cartilage was stained greenish blue (Fig. 2). Figure 3 signifies the findings of the PAS staining method for the detection of lung tissue in studied sausage samples. Carbohydrate ingredients were stained as dim brownish which was considered positive for lung tissue. Figure 4 signifies the findings of the anti-TTF1 immunohistochemical staining method for the detection of lung tissue in the studied sausage samples. The nuclei of pneumocytes and Clara cells were found darker than normal tissues which were considered positive for lung tissue. Figure 5 signifies the findings of the anti-pan-cytokeratin immunohisto-chemical staining method for the detection of lung tissue in studied sausage samples. Positive immunoreactions of airway epithelial cells were considered positive for lung tissue. Figure 6 signifies the findings of the H&E staining of sausage samples to determine the presence of plant structure and striated muscles.

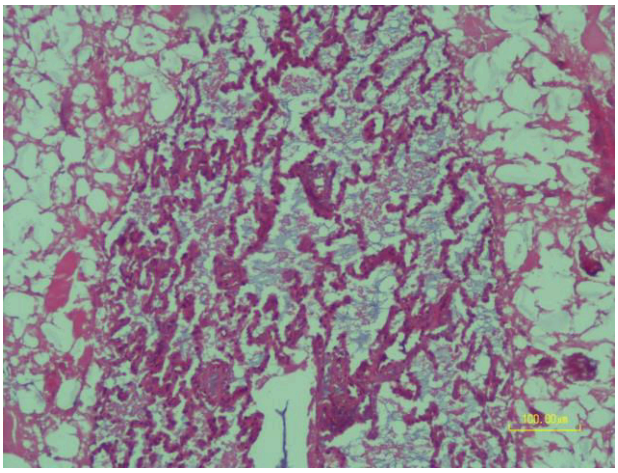

Fig. 1: Group C. This photomicrograph shows a section of lung tissue containing alveolar structures with thin walls, (H&E, scale bar: 100 µm)

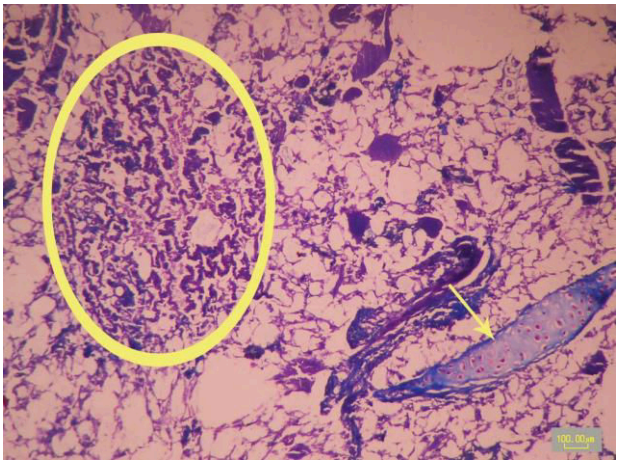

Fig. 2: Group D. Presence of Alveolar structures (circle) and hyaline cartilage (arrow), (Masson trichrome, scale bar: 100 µm)

Comparison of the accuracy of staining techniques

Table 1 signifies the results of the histochemical and

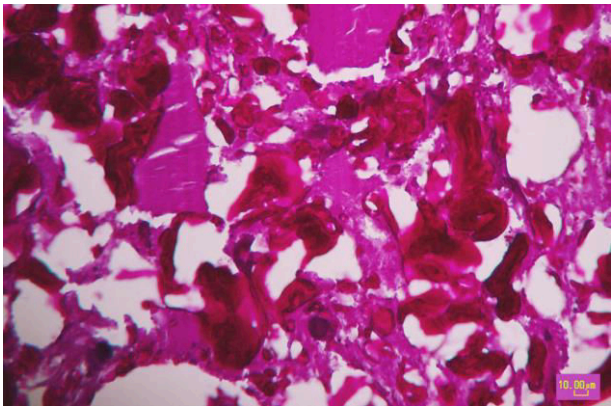

Fig. 3: Group C. Carbohydrate ingredients stained as dim brownish and disappear other tissues, (PAS, scale bar: 10 µm)

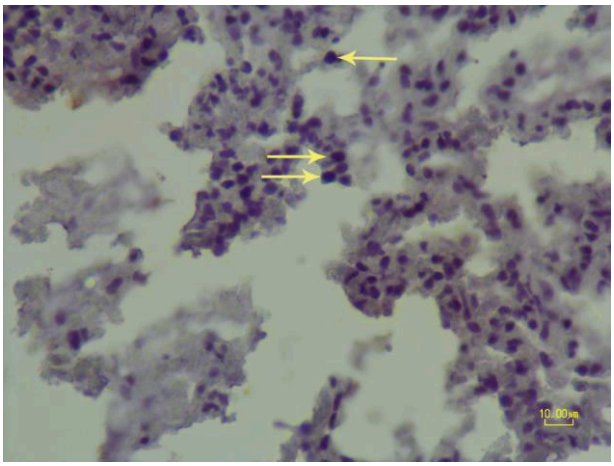

Fig. 4: Group B. The nuclei of pneumocytes and Clara cells are darker than normal ones (arrows), (Anti-TTF1 immunohistochemical staining, scale bar: 10 µm)

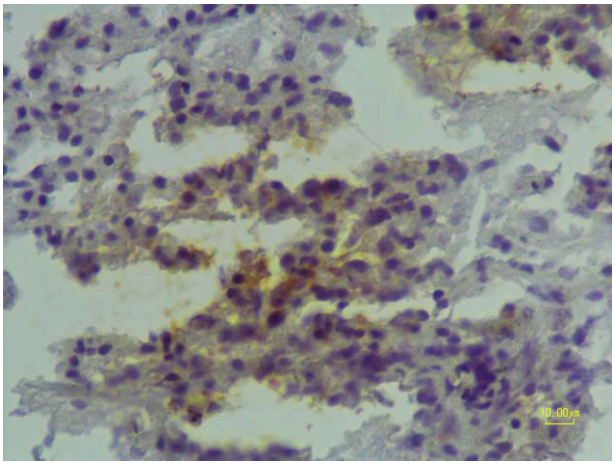

Fig. 5: Group B. Positive immunoreactions of airway epithelial cells related to lung tissue are seen, (Anti-pan-cytokeratin immunohistochemical staining, scale bar: 10 µm)

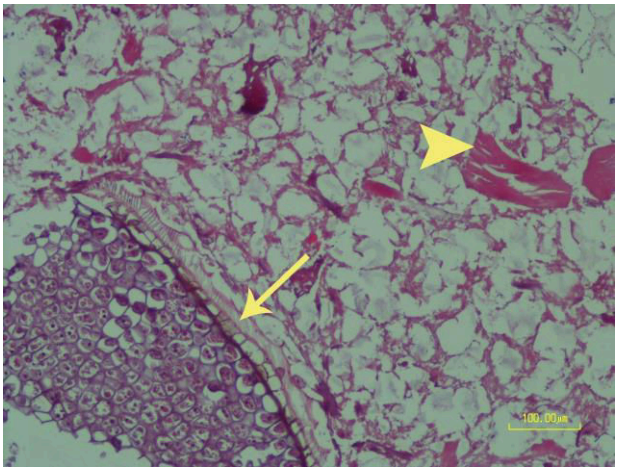

Fig. 6: Group C. Presence of plant structure (arrow) and striated muscles (arrowhead) in the sausage section, (H&E, scale bar: 100 µm)

immunohistochemical techniques in the detection of lung tissue. Among all histochemical tests, Masson trichrome staining had the highest accuracy in the detection of lung tissue in cooked sausage samples. Among two studied methods, TTF1 technique had higher accuracy only in sausage samples treated with 10 g/kg lung tissue. Additionally, TTF1 and Pan-cytokeratin immunohistochemical techniques both detected all contaminated sausage samples treated with 50 g/kg lung tissues. However, Masson trichrome staining cauld detect only 3 contaminated age samples treated with 50 g/kg lung tissues. Table 1 shows the number of positive results in both histochemical and immunohistochemical methods. In Masson trichrome staining, the rate of the lung tissue diagnosed positively, was higher than positive results of another histochemical staining, except for the 50 g/kg added lung tissue in which approximately 60% of the slides stained with H&E and Masson trichrome, were detected positively.

Table 2 signifies the accuracy of different staining methods for detection of lung tissues in studied sausage samples. H&E staining method was considered as a reference. Comparison of the odds ratio of studied techniques revealed that TTF1 staining method had the highest odds ratio (7.4), followed by the Pan-cytokeratin method (6.0). Reversely, PAS staining method had the lowest odds ratio (0.21), followed by Masson trichrome method (1.7). Comparison of the confidence intervale (95%) disclosed that TTF1 method had the highest (1.8-31.0) confidence intervale, while PAS had the lowest (0.02-2.1). In confirmation of the mentioned results in Table 1, the odds ratio of lung tissue detection for Masson trichrome staining was more than other histochemical methods, but this difference was not significant (P>0.05, Table 2).

Table 2: Comparison of the accuracy of different methods for the detection of lung tissue in sausage samples

Detection methods	Odds ratio	CI (95%)	P-value
H&E	1 (Reference)	-	-
Masson trichrome	1.7	0.4-7.3	0.5
PAS	0.21	0.02-2.1	0.2
Anti-TTF1	7.4	1.8-31.0	0.006
Anti-pan-cytokeratin	6.0	1.5-24.6	0.013
Histochemical	1 (Reference)	-	-
Immunohistochemical	7.4	3.0-18.5	0.001

Discussion

Although numerous histochemical studies have been applied to determine whether unauthorized tissues could be detected in the meat products, our results were not in agreement with those of previous studies which reported that histochemical staining was useful to confirm the presence of unauthorized animal tissues in food products (Lazzaro *et al*., 1991; Prayson *et al*., 2008a, b; Sadeghinezhad *et al*., 2015). In the present study, lung structures were seen relatively distinctive using three histochemical staining methods, but in Periodic Acid-Schiff staining, the lung tissues were not detected due to the high contrast of added carbohydrates in sausage which created a dim brownish pattern. Generally, identification of lung tissue by H&E, Masson trichrome and Periodic Acid-Schiff staining in cooked samples was not confirmed. Thus, standard light microscopy was not adequate to detect small autolytic fragments of lung tissue and did not allow discrimination between fragments of mixed materials (a normal component of meat).

According to the Iranian National standard regulations, the use of undesirable organs of slaughtered animals, including the skin, visceral organs, hyaline cartilage, bone, and fat instead of meat in meat products is considered adulteration (Moghtaderi *et al*., 2019). Numerous researches were addressed for disclosure of unauthorized tissues in meat products (Cetin *et al*., 2016; Moghtaderi *et al*., 2019). Investigations on heated meat products demonstrated the presence of adipose tissue, plant material, peripheral nerves, blood vessels, cartilage, bone, gizzard, lymph node, lung tissue, gland tissue, udder tissue, ovary, and cartilage (Latorre *et al*., 2015).

Table 1: Results of the histochemical and immunohistochemical methods for detection of lung tissue

Lung tissue addition (g/kg)	Histochemical methods (positive results/total tested samples)			Immunohistochemical methods (positive results)	
	H&E	Masson trichrome	PAS	TTF1	Pan-cytokeratin
0	0/5	0/5	0/5	0/5	0/5
10	0/5	1/5	0/5	3/5	2/5
25	1/5	2/5	0/5	5/5	5/5
50	3/5	3/5	1/5	5/5	5/5

Immunohistochemical methods are based on the presence of targeting reactive antigens in sections. This technique is reliable and quite sensitive, and therefore it can be used in different aspects of food safety including the detection of food components especially harmful ingredients, the adulteration of foodstuffs and quantification of food ingredients (Kalčáková *et al*., 2021).

In our study, two characteristic protein markers including TTF1 and Pan-cytokeratin were used to detect the lung in cooked samples. Nowadays TTF1 has been considered as a reliable marker to distinguish lung tissue. This antibody is a 38-kDa homeodomain containing DNA-binding protein of the Nkx-2 gene family. It is expressed in epithelial cells in the lung-like type II pneumocytes and Clara cells. The role of TTF-1 in lung involves the regulation of gene expression of surfactant and Clara cell secretory protein (Lazzaro *et al*., 1991; Jagirdar, 2008). Hence in the present research, immunostaining by TTF1 antibody detected TTF1 protein in the nucleus of pneumocytes and Clara cells in alveoli and bronchiole, respectively. Another protein, cytokeratin, is an intermediate filament structural protein, which is found in the cytoskeleton of various epithelial tissues such as airways in lungs (Ring *et al*., 2009). Due to different amounts of added lung tissue, different degrees of staining intensity could be observed. Whereas samples containing 25 g/kg of lung tissue had strong staining, the tissue samples with 10 g/kg lung showed less intensive staining. Cooked sausages without lung tissue revealed no immunoreactions. Generally, positive results obtained by immunostaining by TTF1 seemed to be more accurate than results obtained by Pan-cytokeratin, because the odd ratios of immunohisto-chemical staining by TTF1 was higher than immunohistochemical staining by Pan-cytokeratin. In this way, the immunohistochemical techniques were more reliable in the detection of lung tissue compared to histochemical methods. In another survey (Moghtaderi *et al*., 2019) sausage sections were stained using H&E, Masson's trichrome, Periodic Acid-Schiff/Alcian blue, and Verhoeffe/Van Gieson to detect unauthorized tissues. A wide range of unauthorized tissues was detected, such as dense connective tissue (6.66%), cartilage (28.30%), bone (8.30%), skin (51.60%), smooth muscle (1.66%), and blood vessels (11.66%). They introduced the Masson's trichrome staining as a practical technique for routine assessment of authenticity and quality of sausage to protect the consumers from adulteration. Sadeghi *et al*. (2011) examined 720 sausage samples and found lung unauthorized tissues in about 4% of examined samples using histology. Sadeghinezhad *et al*. (2016) verified the efficacy of MT blue staining for detecting animal and herbal additive tissue in minced meat as well as common H&E staining. Pospiech *et al*. (2009) showed that some special stainings such as the PAS/Calleja staining which targets polysaccharides can indicate soybean flour. Gürbüz *et al*. (2020) reported the higher sensitivity and specificity of immunohisto-chemical methods (AE1/AE3 cytokeratin antibody) compared to histological examination in detection of adulteration in fermented sausage samples.

In agreement with our observation for immunohisto-chemical detection of lung tissue in cooked samples, the result of some studies showed different staining intensities by immunohistochemical detection of CNS in cooked sausages with different levels of brain tissue (Wenisch *et al*., 1999; Tersteeg *et al*., 2002). Also, the presence of spinal cord in Advanced Meat Recovery Systems (AMRS) by immunohistochemical and histochemical staining and polarization microscopy were proved (Kelley *et al*., 2000).

The amount of immunoreaction in food samples is completely based on the state of food (raw, pasteurized and sterilized) and antibody resources even with the same amount of added target tissues (Tersteeg *et al*., 2002). In this regard, one of the most important results of the present study was the ability of antibodies to detect lung tissue in final heated products. This result can be attributed to the heating process in which new epitopes can be generated in samples and used as a new target for new and improved antibodies (Tersteeg *et al*., 2002).

The present study was limited to the lack of the evaluation of the role of molecular techniques for comparison with histological and immunohistochemical methods in the detection of fraud, as well as the lack of evaluation of other commonly used antibodies in the detection of fraud through immunohistochemical tests. Also, due to the absence of raw sausages in Iran, another limitation of this study is the lack of comparison of fraud detection methods on raw sausage samples.

In conclusion, the immunohistochemical procedure has many benefits including less time, less difficulty, and high immunoreaction specificity. Beyond that, histochemical staining can be considered an inefficient method due to the high degree of tissue destruction (Pospiech *et al*., 2011). In the present study, all of the positive samples were detected by immunohistochemical technique compared to histochemical staining, and thus the accuracy of immunohistochemistry makes it very distinctive from conventional and daily pathological staining. Because adding lung tissue to meat products can create a threat for transmission of many diseases from infected animals to humans, national agencies need to apply more strict controls and surveillance and improve their hazard analysis and critical control points.

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Conflict of interest

The authors declare that they have no conflict of interest.

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