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Sushma
Ph.D. Scholar, Department of Veterinary Pathology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

Vikas Nehra
Scientist, Department of Veterinary Pathology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

Deepika
Assistant Professor, Department of Veterinary Pathology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

Pooja Kundu
Ph.D. Scholar Department of Veterinary Public Health and Epidemiology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

Correspondence
Sushma
Ph.D. Scholar, Department of Veterinary Pathology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India

Histochemical staining procedures for differentiation of tissue/cell components in lambs and kids carcasses

Sushma, Vikas Nehra, Deepika and Pooja Kundu

Abstract
Study was conducted on total of 25 carcasses including nine kids and sixteen lambs of six month age showing respiratory and digestive disturbances brought to the Department of Veterinary Pathology, LUVAS, Hisar during the period of seven months. The pathological lesions were mainly noticed in intestine, liver, abomasum, mesenteric lymph nodes, spleen, lungs, heart and kidneys. For further differentiation and characterization of the lesions the formalin fixed sections were stained by the various standard special tissues staining techniques viz. Taylor's modified Brown and Brenn method for Gram's character of bacterial colonization, PAS-Alcian blue staining for mucosubstances and Pearl's staining for haemosiderin. In enteritis affected carcasses, intestine revealed goblet cell hyperplasia showing blue coloured mucosubstances with PAS-Alcian blue staining. In cases of pnuemonia, Taylor's staining, section of the lungs revealed the presence of bright red coloured rod shaped gram negative bacteria. In different sections of spleen with severe hemorrhgaes, Perl's staining revealed presence of haemosiderosis in red pulp area. These differential staining methods may provide a valuable and cost-effective tool for the diagnostic histopathology and for the researchers in histology.

Keywords: brown and brenn method, pas-alcian blue, pearl's, taylor's staining

1. Introduction
Histology is the microscopic study of animal and plant cell and tissues through staining and sectioning and examining them under a microscope (Electron or light microscope). There are various methods used to study tissue characteristics and microscopic structures of the cells. Histological studies are used in forensic investigations, autopsy, diagnosis and in education. In addition, histology is used extensively in medicine especially in the study of diseased tissues to aid treatment (Black, 2012) [2].
Histological staining is a series of technique processes undertaken in the preparation of sample tissues by staining using histological stains to aid in the microscope study (Anderson, 2011) [1]. The process of histological staining takes five key stages which involves fixation, processing, embedding, sectioning and staining (Titford, 2009) [13]. Great changes have been done regarding techniques used for histological staining through chemical, molecular biology assays and immunological techniques collectively and these have greatly facilitated in the study of organs and tissues (Shostak, 2013) [11].
There are hundreds of special stains in use, each with its own unique properties, which can help to evaluate certain cellular or tissue components, and even demonstrate the presence of pathogens. Although these stains play a very important role in histology, they are best used to confirm a suspected finding after evaluation of H & E-stained tissue sections, rather than being used in isolation to make a diagnosis.

2. Materials and Methods
2.1 Source of study and different samples collected
Post-mortem examination on 25 carcasses of young ruminants (lambs and kids) up to the age of six months brought to Department of Veterinary Pathology, LUVAS, Hisar was conducted during the period of seven months (September, 2015 to March, 2016). Out of 25 carcasses, 16 cases were of lambs and 9 of kids. Thorough post-mortem examination on the carcasses of lambs and kids were conducted for examining pathological lesions and appropriate tissues from organs such as intestine, stomach part, liver, lung, trachea, heart and mesenteric lymph nodes were collected in 10% buffered formalin for special staining procedures.

2.2 Special staining procedures

2.2.1 Taylor's staining

On histopathological examination, the section showing bacterial colonization were stained by the technique suggested by Taylor (1966) [12], which is the modification of Brown and Brenn (1931) [3] method for the demonstration of bacteria in the tissue sections. In this technique, Gram positive organisms stained blue to blue black whereas Gram negative bacteria are stained bright red in colour. Nuclei, erythrocytes, necrotic tissue, cytoplasm and connective tissue took brownish red, red to greenish, yellowish green, yellow and red colour respectively.

2.2.2 Procedure

The tissues were fixed in formalin and paraffin sections cut at 3-4 µ and then deparaffinise and hydrate to distilled water. These sections were then kept in Harris haematoxylin solution for 5-10 min. Subsequently, washed in running water for 1 min and differentiated in acid alcohol solution and again washed in running water for 3 min. After this, the sections were washed in saturated lithium carbonate solution to intensify the blue colour. From this point, we carry only one slide at a time and washed the same in running water for 5 min, in Hecker's solution for 2 min and then washed quickly in water. Mordant in Gram's iodine solution was added for 1 min and then washing of the slide was done with water. Slide was blotted (but was not allowed to dry) using filter paper moistened with water. The slide was than decolorized in ethyl ether-acetone until no more blue colour comes off. Blotting was performed again (but without drying) using filter paper moistened with ethyl ether-acetone. Subsequently, working basic fuschin solution was added for 3 min. Again, blotting was done using filter paper (again drying was avoided). After this, slide was dipped in acetone until sections begin to decolorize and was passed quickly to picric acid-acetone solution to decolorize and differentiate until section becomes reddish brown-yellow (this was done for approximately 15 sec). Finally, the slide was passed quickly through acetone and xylene solution I, then acetone and xylene solution II and cleared in two changes of xylene. In the end mounting of slide was done with DPX.

2.3 Pearl's staining for haemosiderin demonstration

On histopathological examination, H & E stained sections of spleen and lungs showing presence of haemosiderin were also stained by the Perl's staining technique (Luna, 1968) [7]. In this technique, Ferric iron is stained bright blue, nuclei as red and cytoplasm as light pink in colour.

2.3.1 Procedure

The tissues were fixed in formalin and paraffin sections of 3-4 µ were cut and then deparaffinised and hydrated to distilled water. These sections were kept in stock potassium ferrocyanide solution for 5 min and subsequently in working potassium ferrocyanide-hydrochloride solution for 20 min. These sections were rinsed well in distilled water and counterstaining in nuclear fast red solution was done for 5 min. After this sections were washed well in running water and dehydrated in 95 % alcohol, absolute alcohol and finally cleared in xylene (two changes each). At the end mounting of the sections was done in DPX.

2.4 PAS-Alcian blue staining for mucosubstances

On histopathological examination, the section of intestine showing presence of goblet cell hyperplasia on H & E staining were also stained by PAS- Alcian blue (pH 2.5) staining for showing the presence of mucosubstances (Luna, 1968) [7]. Mucins are complex carbohydrates secreted by different types of epithelial cells and glandular tissues of gastrointestinal tract. Mucins are mainly of two types-Neutral and Acidic (Nikumbh *et al*., 2012) [9]. All polysaccharides and mucosubstances containing hexoses or deoxyhexoses with vicinal glycol groups stain magenta or red. Those mucosubstances staining red include neutral muccosubstances, hyaluronic acid, sialomucins and most strongly acidic sulphated mucosubstances stain blue.

2.4.1 Procedure

The tissues were fixed in formalin and paraffin sections were cut at 3-4 microns and then deparaffinized and hydrated with distilled water. Alcian blue solution (pH 2.5) was added for 30 min. Subsequently, sections were washed in running water for 5 min, oxidized in periodic acid solution for 5 min, washed in running water for 5 min. Schiff reagent solution was added for 10 min and rinsed in sodium metabisulfide solution (three changes) for 2 min each. Sections were washed in running tap water for 10 min and dehydrated in 95 % alcohol, absolute alcohol and cleared in xylene (two changes each). In the end sections were mounted in DPX.

3. Results and Discussion

Main pathological lesions observed in intestine of lambs were vascular congestion (11 cases) and catarrhal enteritis characterised by presence of catarrhal exudate (3 cases) on mucosa and microscopically by infiltration of leucocytes in lamina propria and degeneration and desquamation of mucosal epithelium and glands. Catarrhal type inflammation revealed goblet cell hyperplasia having mucosubstances which was further confirmed by PAS-Alcian blue staining which gives blue colour which is positive for acidic sulphated mucin. Similar type lesions were also noticed in kids, where the main pathological lesions observed in intestine were congestion (5 cases), haemorrhages (2 cases) and presence of catarrhal exudate (1 case) in intestine indicating catarrhal enteritis (Fig.1-2). These results are in accordance with the findings of other researchers (Shah and Shrikhande, 1989; Nikumbh *et al*., 2012) [9]. From a histochemical point of view, mucins can have two broad categories based upon the chemistry and composition of the carbohydrate component of the mucin. The charged or "acid" mucins contain carbohydrates with carboxylate (COO-) or -sulphonate (SO_3) groups. Both of these groups are ionized at a physiologic pH to produce an overall negative charge on these mucin molecules. The carbohydrate chains of neutral mucins lack acidic groups and thus carry no net charge (Myers, 2019) [8]. The acid mucins are found widely distributed throughout the gastrointestinal tract and the respiratory tract and appear as blue in colour with PAS-Alcian blue staining. Whereas, the neutral mucins can be found primarily in the surface epithelia of the stomach, Brunner's glands of the duodenum and in the pro-static epithelium. The combination of the alcian blue and the PAS techniques can be used as a means of distinguishing neutral mucins from acid mucins. In most protocols, sections are stained with the standard alcian blue (pH 2.5) method followed by the PAS technique. The alcian blue at a pH of 2.5 will stain all acid mucins deep blue but will not color the neutral mucins. The subsequent application of the PAS technique will stain the neutral mucins bright magenta.

Tissues or cells that contain both neutral and acidic mucins may demonstrate a dark blue or purple coloration. The combined application of alcian blue and PAS is useful for several reasons. Changes in the distribution or pattern of expression of neutral and acid mucins are indicative of certain pathological conditions. In addition, the combined alcian blue/PAS technique is perhaps the most sensitive or comprehensive means for detection of mucins as all mucins should react regardless of the charge nature of the mucin (Myers, 2019) [8].

Grossly in lungs of lambs, most prominent changes were congestion (6 cases) followed by pneumonic consolidation (2 cases) and suppurative pneumonia (1 case) characterised by presence of seropurulant exudate in alveoli, infiltration of leucocytes and emphysema. Upon Taylor's staining of the exudate, rod shaped Gram negative bacteria (bright red coloured) were noticed (Fig. 3). Upon microbiological isolation and identification these bacteria were confirmed to be *Salmonella* spp. Pathological lesions in lungs of kids, were congestion (4 cases) and serous pneumonia (1 case) characterised by presence of serous fluid in alveoli along with infiltration of leucocytes and emphysema and presence of rod shaped Gram negative bacteria (bright red coloured) in Taylor's staining. Upon microbiological isolation and identification these bacteria were found to be *E. coli* and *Proteus* spp. Earlier workers Lehreena *et al*. (2010) [6] also reported red rod shaped bacilli in the exudate and in the vicinity of alveolar epithelium in buffalo calves upon Taylor's stain of sections from lungs affected with interstitial pneumonia. They also reported presence of Gram negative rod shaped bacilli in the section of different cases of hepatitis by Taylor's stain indicating *E. coli.* Hepatic lesions of similar lesions have also been described by Jubb *et al*. (1985) [5] in calves due to colibacillosis. In spleen of kids, brown coloured deposits suspected of haemosiderosis were noticed which were confirmed as iron containing hemosiderin on Perl's staining (Fig. 5). Hemosiderin is a breakdown product of haemoglobin and is thought to be composed of ferric iron and protein. It may be present in tissues in certain pathologic conditions such as hemochromatosis. This yellow-brown pigment is insoluble in alkalis and water but is soluble in acid even after fixation. Treating a tissue section with 10% sulphuric acid overnight will usually remove this pigment. Perl's staining can be used especially for hemochromatosis as well as hemosiderosis primarily in liver and spleen as excess iron deposition is stained as blue granules. It can also be used for the diagnosis of gastric ulcer produced due to iron overdoses (Churukian, 2009) [4].

It can be concluded from the present study that histochemical staining methods for mucin staining, Taylor's staining and Perl's staining may provide a valuable and cost-effective tool for the diagnostic histopathology and for the researchers in histology. Also, mucin histochemistry particularly PAS-Alcian blue staining can effectively determine the presence and types of mucins i.e. neutral or acidic mucin.

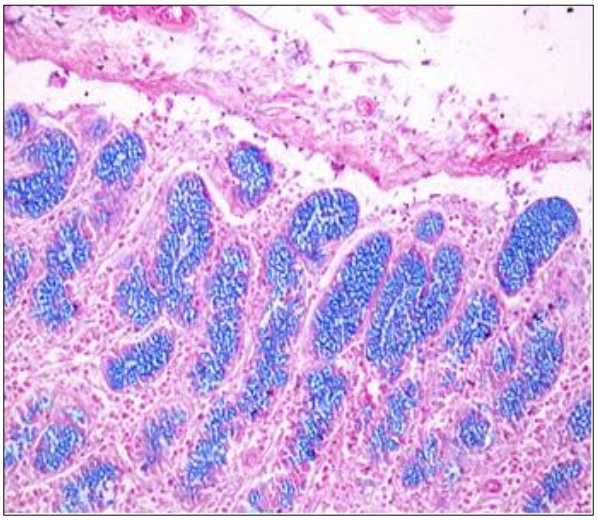

Fig 1: Intestine of kid showing blue coloured mucosubstances (due to Goblet cell hyperplasia in catarrhal enteritis) with PAS-Alcian blue staining (pH 2.5) staining, (H&E X200)

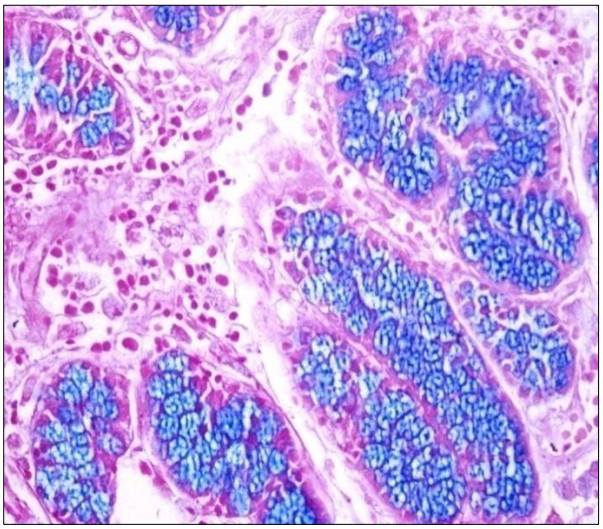

Fig 2: Intestine of kid showing blue coloured mucosubstances (due to Goblet cell hyperplasia in catarrhal enteritis) with PAS-Alcian blue staining (pH 2.5) staining (Higher magnification of Fig. 1), (H&E X400)

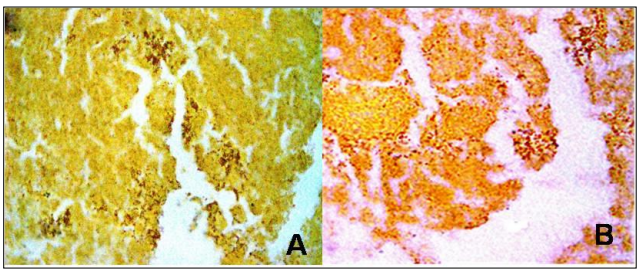

Fig 3(A&B): Lungs of lamb showing presence of rod shaped Gram negative *Salmonella* spp. bacteria (bright red coloured) in Taylor's staining along with suppurative pneumonia, (H&E X1000)

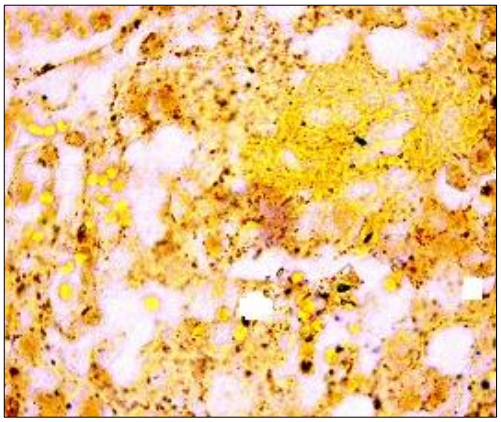

Fig 4: Lung of kid showing presence of rod shaped Gram negative *E. coli* and *Proteus* spp. bacteria (bright red coloured) along with serous pneumonia with Taylor's staining, (H&E X1000)

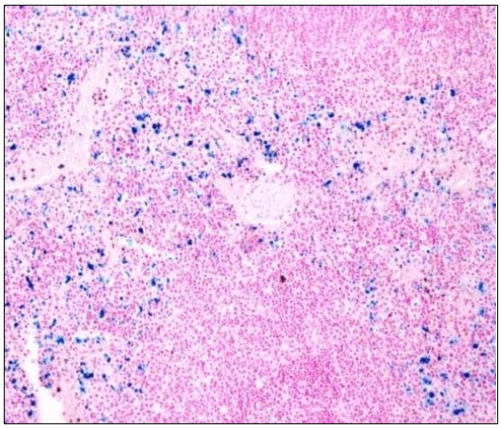

Fig 5: Spleen of kid showing presence of haemosiderosis in red pulp area with Pearls' staining, (H&E X100)

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Conflict of Interest
Authors declare that there is no conflict of interest regarding the present research work.

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