AATCC Review

27 July 2024: Received
28 August 2024: Revised
05 October 2024: Accepted
06 November 2024: Available Online

https://aatcc.peerjournals.net/

Original Research Article

Open Access

Gross, Histomorphometrical and Histochemical studies on the gizzard of indigenous poultry of Poonch region of Jammu and Kashmir, India

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ABSTRACT

The present research was conducted on the gizzard of 06 indigenous poultry of the Poonch region. The gizzard wall consisted of tunica mucosa containing mucosal glands, tunica submucosa, tunica muscularis and tunica serosa with an additional koilin layer. Lamina epithelialis consisted of simple columnar epithelium. Lamina propria contained gastric glands which were simple tubular branched in cranial and middle parts of the gizzard whereas simple tubular glands in the caudal part. Lamina muscularis mucosae was absent except at few locations towards the caudal part of the gizzard. The koilin layer, epithelium, lamina propria submucosa and tunica muscularis were significantly thicker in the middle part of the gizzard. The koilin layer, epithelium of mucosal folds and secretory material within the lumen of glands showed positive PAS reaction. Superficial glands showed strong PAS reaction whereas the deeper glands showed a positive reaction for Alcian Blue. The koilin layer showed a strong reaction for basic proteins and a moderate reaction for lipids.

Keywords: *Gizzard, Glands, Koilin, Histomorphology, Histochemistry*

Introduction

The Union Territory of J&K belongs to the greater Himalayan mountain range which exerts significant influence on its agro-climatic conditions. District Poonch has a humid, subtropical climate which is much cooler than the rest of India due to its moderately high elevation and northerly position. Indigenous poultry rearing provides sustainability to the local people. The local Poonchi bird weighs about 2.1-2.5 kg (cock) and 1.6-1.8 kg (hen). Females are combless whereas males present red coloured comb and can survive well in 0°C temperature.

The digestive system consists of the organs responsible for food absorption, chemical digestion, and mechanical reduction. The stomach, which is made up of two parts, the muscular stomach (gizzard), which is situated caudal to the proventriculus, and the glandular portion (proventriculus), is the most active component of the digestive system of birds [1]. A gizzard is crucial for grinding food because it is bigger and more powerful than the proventriculus [1]. The gizzard's grinding function prepares the food for subsequent digestion in the gut [2].

Although the literature is available on the histomorphology of gizzard of red jungle fowl [3], Black-tailed crake [4], guinea fowl [5], dove [6], Iraqi falcon [7], broiler chicken [8], adult starling bird [9], Japanese quail [10]. Micrometrical studies have been done on the gizzard of Kadaknath fowl [11]. Data is also available on the histochemical studies of gizzard in hawk, crow and sparrow [12], Black-tailed crake [4], Iraqi falcon [7], broiler chicken [8]. Data is also available on the proventriculus of indigenous poultry of the Poonch region [13]. No data is available on the histomorphometry and histochemistry of gizzard of local poultry of Poonch region. Therefore, this study was planned to generate information on the histomorphology and histochemistry of the gizzard of the local poultry of Poonch region. The study will further help to unveil the distribution and localization of various histochemical components.

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DOI: https://doi.org/10.21276/AATCCReview.2024.12.04.206

Materials and Methods

Sample collection: The carcass of 06 indigenous birds of the Poonch region was obtained from the Division of Animal Genetics and Breeding, F.V.Sc & A.H., SKUAST-Jammu. Immediately after collection, body weight and weight of gizzard were recorded using Monopan balance and fixed in 10% Neutral Buffered Formalin for 24 hours.

Fixation and processing of tissue for light microscopy: Immediately after collection, the tissue samples were fixed in 10% Neutral Buffered Formalin (NBF). After proper fixation, the tissue samples were washed, dehydrated, and cleared by standard tissue processing schedule [14]. The tissue samples were embedded in paraffin wax (58-60 °C) for paraffin block preparation. For histomorphology, tissue samples were taken from three sites i.e. cranial (G1), middle (G2), and caudal (G3) (Fig. 1). Tissue samples were processed and sections of 5 µ thickness were obtained. The sections were subjected to various histological and histochemical methods as detailed hereunder.

1. Hematoxylin and Eosin stain for routine histomorphology
2. Van Gieson & Verhoeff's stain for collagen and elastic fibers
3. Gomori's method for reticular fibers
4. Periodic Acid Schiff's method for neutral mucin
5. PAS-AB (pH 2.5) for neutral and acid mucin
6. Bromphenol Blue for basic proteins
7. Sudan Black B for lipids

Micrometrical parameters: Different micrometrical parameters were recorded:

a. Thickness of Kaolin layer (µ)

b. Epithelium thickness (μ)
c. Thickness of Lamina propria-submucosa (μ)
d. Thickness of Tunica muscularis (μ)
e. Thickness of Tunica serosa (μ)
f. Glandular diameter (μ)

Statistical analysis: The results were presented as Mean ± Standard Error. The data was subjected to standard statistical analysis [15].

Results

Gross anatomy: The gizzard (muscular stomach) was located immediately succeeding the proventriculus and was somewhat spherical (Fig. 1). It was placed partly between the two lobes of the liver and mostly covered by the left lobe of the liver. It was built with thick strong muscles. The average antero-posterior length was 5.142 ± 0.19 cm whereas the transverse width was 5.266 ± 0.14 cm.

Histomorphology: The tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa were the four layers that made up the gizzard wall (Fig. 2). In addition to these four tunics, an additional internal secretory layer known as koilin was present above tunica mucosa (Fig. 2) towards the luminal surface.

Tunica mucosa contained lamina epithelialis, lamina propria and lamina muscularis mucosae. Mucosal folds of different shapes lined with lamina epithelialis were seen which were widely separated from one another by the spicules of the koilin layer (Fig. 3). Lamina epithelialis was simple columnar with an oval nucleus located towards the base and basophilic cytoplasm (Fig. 4). The cells towards the tip of mucosal folds appeared taller.

Lamina propria had gastric glands distributed in loose connective tissue rich in thick collagen fibers, fine reticular and elastic fibers. These fibers extended in between the glands (Fig. 5 and 6). The glands were simple tubular branched in the cranial (G1) and middle (G2) parts of the gizzard whereas, in the caudal part (G3), simple tubular glands were seen with occasional branching (Fig. 6). The lumen of the glands was filled with homogenous eosinophilic secretions which were more pronounced in the middle (G2) part of the gizzard (Fig. 7).

Lamina muscularis mucosae was absent except at a few locations towards the caudal (G3) part of the gizzard where it was reinforced with collagen, reticular and elastic fibers. As a result, tunica submucosa merged with lamina propria forming lamina propria submucosa having connective tissue fibers extending towards lamina propria as well as towards tunica muscularis layer.

The mucosal gland's secretory product lining the luminal surface solidifies to form hard cuticle known as koilin. In the present study, the koilin layer was oriented in two forms (Fig. 3). The horizontal part was thick with irregular luminal surface having tooth-like processes. The irregular luminal surface was more noticeable at the cranial part of the gizzard as compared to the middle and caudal parts. The vertical layer was thin and had a cone- or spicules-like arrangement that projected in between the folds of mucosa forming finger-like projections.

Tunica muscularis was well developed. At the aG1 level (Cranially), inner circular and outer longitudinal muscle layers were observed (Fig. 8A). In between these muscle bundles, coarse collagen fibers were seen reinforcing the muscle layer. In the middle region (G2), only a single layer of circular smooth muscle was evident which was surrounded by a thick tendinous layer of collagen bundles. The branching pattern of the circular muscle layer was also evident at few places (Fig. 8B). Caudally (G3), the inner oblique and outer circular layer of smooth muscle bundles were observed (Fig. 8C). The circular muscle layer alternated and intermingled with fine collagen and elastic fibers. Tunica serosa consisted of loose connective tissue containing blood vessels and nerves and covered by mesothelium (Fig. 2).

Micrometry: The micrometrical parameters were recorded from three sites, i.e. cranial (G1), middle (G2), and caudal (G3) part of the gizzard (Table 1). The thickness of the koilin layer was significantly higher at G2 (424.37 ± 17.26 μ) followed by G3 (247.73 ± 26.28 μ) and least in G1 (186.34 ± 8.54 μ).

The thickness of epithelium was also significantly higher at G2 (444.32 ± 44.25 μ) followed by G1 (297.14 ± 11.69 μ) and least in G3 (271.78 ± 17.63 μ). The thickness of lamina propria-submucosa was significantly higher at G2 (310.42 ± 38.65 μ) followed by G3 (102.42 ± 4.19 μ) and least at G1 (84.65 ± 5.21 μ).

The thickness of tunica muscularis was significantly higher at G2 (1518.97 ± 144.90 μ) followed by G1 (1099.04 ± 122.10 μ) and least at G3 (836.14 ± 92.22 μ). The thickness of tunica serosa was 105.35 ± 9.62 μ at G1, 107.91 ± 5.19 μ at G2, and 119.83 ± 21.11 μ at G3. The difference was statistically non-significant.

The glandular diameter was 35.70 ± 3.59 μ, 32.73 ± 1.99 μ and 34.09 ± 2.52 μ at G1, G2 and G3, respectively. However, the difference was non-significant statistically. The height of glandular epithelium at G1, G2, and G3 was 7.61 ± 0.60 μ, 7.16 ± 0.36 μ, and 7.70 ± 0.30 μ, respectively, although non-significant statistically.

Histochemistry: The koilin layer showed a strong positive reaction for PAS (Fig. 9). The epithelial lining of the mucosal folds showed a strong positive PAS reaction. The luminal secretions of tubules also showed a strong PAS-positive reaction (Fig. 10). The connective tissue in lamina propria submucosa and tunica muscularis showed moderate PAS reaction. Smooth muscle fibers gave a mild PAS reaction.

With combined PAS-AB (pH 2.5), the koilin layer showed pink colored positive PAS reaction with patches of Alcian Blue reaction (Fig. 11). The mucosal lining epithelium stained strongly positive for both PAS and AB (pH 2.5) (Fig. 11). It indicated the presence of both neutral and acid mucins. The connective tissue gave a mild reaction for PAS but was found negative for the Alcian Blue stain.

The koilin layer showed a strong reaction for basic proteins with Bromphenol Blue stain whereas the muscle layer showed a moderate reaction for basic proteins (Fig. 12). The outer surface of koilin layer showed a moderate reaction for lipids (Fig. 13).

Discussion

Gross anatomy: The gizzard (muscular stomach) was located immediately succeeding the proventriculus and was somewhat spherical. It was placed partly between the two lobes of the liver and mostly covered by the left lobe of the liver similar to the observation made in dove [6]. The ostrich's gizzard was biconvex lens shape [16]. The average antero-posterior length was 5.142 ± 0.19 cm whereas the transverse width was 5.266 ± 0.14 cm which was higher than values recorded in other species. The gizzard of an adult starling bird was 3.22 cm in length [9]. The length and width of the gizzard of ostrich were 26.45 ± 0.61 cm and 16.72 ± 0.31 cm, respectively [16].

Histomorphology: The wall of the gizzard was made of four layers namely the tunica mucosa, tunica submucosa, tunica muscularis, and tunica serosa as earlier reported in *Rhynchotus rufescens* [17] and *Struthio camelus* [18]. An additional internal secretory layer known as koilin was present above the tunica mucosa towards the luminal surface similar to the observation made in guinea fowl [5].

Tunica mucosa contained lamina epithelialis, lamina propria, and lamina muscularis mucosae. Mucosal folds of different shapes lined with lamina epithelialis were seen which were widely separated from one another by the spicules of the koilin layer. Lamina epithelialis was simple columnar with an oval nucleus located towards the base and basophilic cytoplasm. The cells towards the tip of mucosal folds appeared taller. Similar observations were made in guinea fowl [19] and broiler chicken [8]. However, in mallard [20] and domestic fowl [21] simple cuboidal epithelium was observed.

Lamina propria had gastric glands distributed in loose connective tissue rich in thick collagen fibers, fine reticular and elastic fibers. These fibers extended in between the glands similar to the findings in guinea fowl [5]. The glands were simple tubular branched in the cranial (G1) and middle (G2) parts of the gizzard whereas in the caudal part (G3), simple tubular glands were seen with occasional branching. The lumen of the glands was filled with homogenous eosinophilic secretions which were more pronounced in the middle (G2) part of the gizzard as earlier observed in Japanese quail [1]. Branched tubular glands were seen in Japanese quail [1]. In guinea fowl, simple straight branched tubular glands were reported by Selvan *et al* [5] and long tubular glands were reported by Saran and Meshram [22]. In Red Jungle fowl [23] and Moorhen [24], simple tubular glands were observed.

Lamina muscularis mucosae was absent except at few locations towards the caudal (G3) part. As a result, tunica submucosa merged with lamina propria forming lamina propria submucosa having connective tissue fibers extending towards lamina propria as well as towards tunica muscularis layer similar to the findings of in Red Jungle fowl [23], domestic fowl [25] and Japanese quail [10]. However, in mallard [20], starling bird [9], moorhen [24], and kestrel [25], the lamina muscularis mucosae layer was present.

The mucosal gland's secretory product lining the luminal surface solidified to form hard cuticle known as koilin. According to King and Mclelland [26], the gizzard was lined with keratin-like layer of koilin known as cutical gastric. Konishi [27] observed that cuticle is softer in composition in birds with thin sac-like gizzard. The present study contradicted the findings of Alsheshani [28] who mentioned that the koilin layer was absent in the gizzard of Accipiter nisus. In the present study, koilin layer was oriented in two forms. The horizontal part was thick with an irregular luminal surface having tooth-like processes, which were designated as dentate processes by Selvan *et al* [5] in guinea fowl. The irregular luminal surface was more noticeable at the cranial part of gizzard as compared to the middle and caudal parts. The vertical layer was thin and had a cone- or spicules-like arrangement that projected in between the folds of mucosa forming finger-like projections. The presence of the koilin layer was also observed in guinea fowl [5], and pigeon [29]. The koilin layer served as a substitute for the absence of teeth in the birds [30].

At the G1 level (Cranially), inner circular and outer longitudinal muscle layers were observed. In the middle region (G2), only a single layer of circular smooth muscle was evident which was surrounded by thick tendinous layer of collagen bundles. The branching pattern of the circular muscle layer was also evident in few places. Caudally (G3), inner oblique and outer circular layer of smooth muscle bundles were observed. In Japanese quail, most of the smooth muscle fibers were arranged circularly with few fibers arranged as an outer longitudinal and inner oblique layer [1]. However, in mallards [20], broiler chickens [8] and Red Jungle fowl [23], three layers of muscles were arranged as inner, outer longitudinal and very thick intermediate circular layer. In Coot birds [31], most avian species [32], ducks [33] and ostrich [16], only two layers of muscle fibers in gizzard were reported which were arranged as outer longitudinal and inner circular.

Tunica serosa layer consisted of loose connective tissue containing blood vessels and nerves and covered by mesothelium which was similar to the observation made in Mallard [20].

Micrometry: The thickness of the koilin layer was significantly higher at G2 followed by G3 and least in G1. The thickness of the inner cornified layer of gizzard in day-old, 7, 28 and 112 days old Kadaknath birds was 184.5 ± 18.595 µ, 276 ± 20.666 µ, 368 ± 15.567 µ and 411 ± 23.872 µ, respectively [11]. In Japanese quail, the thickness of the koilin layer was 318.63 ± 127.66 µ [10].

The thickness of epithelium was also significantly higher at G2 (444.32 ± 44.25 µ) followed by G1 (297.14 ± 11.69 µ) and least in G3 (271.78 ± 17.63 µ). The thickness of lamina propria-submucosa was significantly higher at G2 (310.42 ± 38.65 µ) followed by G3 (102.42 ± 4.19 µ) and least at G1 (84.65 ± 5.21 µ). The thickness of the tunica mucosa of gizzard in day-old, 7, 28 and 112days old Kadaknath birds was 300.5 ± 12.028µm, 319 ± 11µm, 459 ± 27.826 µm, and 634 ± 26.381 µm respectively [11].

The thickness of tunica muscularis was significantly higher at G2 followed by G1 and least at G3. The thickness of tunica muscularis in guinea fowl ranged between 342.5 ± 6.7 µ to 446.4 ± 4.1 µ at day old and 12 weeks of age [5]. In adult starling bird, the thickness of tunica muscularis was 3256 µ [9]. In Japanese quail, the thickness of tunica muscularis was 1480.07 ± 739.43 µ [10].

Tunica serosa was thickest at G3 but the difference was statistically non-significant. The thickness of the tunica serosa of gizzard in day-old, 7, 28 and 112-old Kadaknath birds was 23.1 ± 2.350 µm, 36.15 ± 3.582 µm, 50.25 ± 3.598 µm, and 81.5 ± 9.945 µm, respectively [11]. In adult starling birds, the thickness of tunica muscularis was 118 µ [9]. In Japanese quail, the thickness of tunica serosa was 60.44 ± 30.29 µ [10].

The glandular diameter was maximum at G1 but the difference was non-significant statistically. Similarly, the height of glandular epithelium did not show any significant difference at G1, G2 and G3. The height of gizzard glands epithelium of Japanese quail during 1, 15, 30, 45 days was 6 ± 2.12, 9 ± 1.90, 14 ± 2.43, and 17 ± 1.90 µm respectively [1]. In Kadaknath birds, the average height of the epithelial cell of the gizzard mucosa in day-old, 7, 28 and 112-days old birds was 7.62 ± 0.471 µm, 7.7 ± 0.478 µm, 9.35 ± 0.605 µm, and 10.6 ± 0.515 µm respectively [11].

Histochemistry: The koilin layer showed a strong positive reaction for PAS similar to the findings in black-winged kite [32], mallard [20] and moorhen [24]. The epithelial lining of the mucosal folds showed strong positive PAS reaction as also observed in broiler chicken [8] and moorhen [24]. The luminal secretions of tubules also showed a strong PAS-positive reaction.

These results were similar to the observations made in kite [32] and Iraqi falcon [7]. However, Al-Saffar and Al-Samawy [20] in Mallard and Taher *et al* [24] in Moorhen reported negative reaction with PAS. The connective tissue in lamina propria submucosa and tunica muscularis showed moderate PAS reaction. Smooth muscle fibers gave mild PAS reaction similar to the findings in Mallard [20].

With combined PAS-AB (pH 2.5), koilin layer showed pink-colored positive PAS reaction with patches of Alcian Blue reaction. Zhu [4] in Black-tailed crake reported that koilin layer showed positive reaction for PAS but showed positive reaction for AB (pH 2.5) only on its interior surface. Al-Saffar and Al-Samawy [20] in Mallard and Taher *et al* [24] in Moorhen reported negative AB reaction of koilin layer. PAS positive koilin layer was also observed in guinea fowl [5]. The mucosal lining epithelium stained strongly positive for both PAS and AB (pH 2.5) similar to the findings in Mallard [20] and broiler chicken [8]. It indicated the presence of both neutral and acid mucins. The presence of neutral and acid mucin may protect the mucosal surface [32]. The connective tissue gave a mild reaction for PAS but was found negative for the Alcian Blue stain.

The koilin layer showed a strong reaction for basic proteins with Bromphenol Blue stain whereas the muscle layer showed moderate reaction for basic proteins. The outer surface of the koilin layer showed a moderate reaction for lipids.

Table 1 Showing different micrometrical parameters of gizzard of Indigenous poultry of the Poonch region

Parameter (µ)	Cranial (G1)	Middle (G2)	Caudal (G3)
Thickness of Kaolin	186.34 ± 8.54[a]	424.37 ± 17.26[b]	247.73 ± 26.28[c]
Thickness of Epithelium	297.14 ± 11.69[a]	444.32 ± 44.25[b]	271.78 ± 17.63[a]
Thickness of Lamina Propria-submucosa	84.65 ± 5.21[a]	310.42 ± 38.65[b]	102.42 ± 4.19[a]
Thickness of Tunica muscularis	1099.04 ± 122.10[a]	1518.97 ± 144.90[b]	836.14 ± 92.22[a]
Thickness of Tunica serosa	105.35 ± 9.62[a]	107.91 ± 5.19[a]	119.83 ± 21.11[a]
Glandular diameter	35.70 ± 3.59[a]	32.73 ± 1.99[a]	34.09 ± 2.52[a]
Height of glandular epithelium	7.61 ± 0.60[a]	7.16 ± 0.36[a]	7.70 ± 0.30[a]

Mean values with the same superscript within the row do not differ significantly (p>0.05)

Conclusion

The gross, histomorphological and histochemical studies were carried out on the gizzard of indigenous poultry of the Poonch region. The present study demonstrated that the histological architecture of the gizzard of indigenous poultry of the Poonch region and the distribution of its mucins resembled those of other avian species. Some of the variations may be associated with its diet and nutritious behavior.

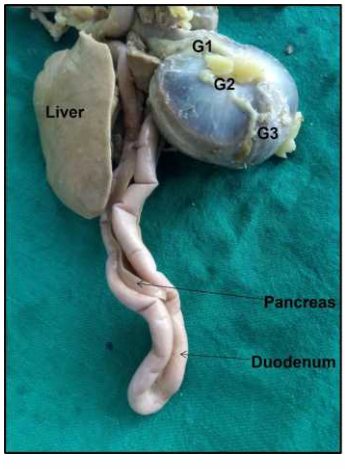

***Fig. 1** Photograph showing the sites from which tissue samples were collected i.e. cranial (G1), middle (G2) and caudal part (G3)*

***Fig. 2** Photomicrograph of gizzard (G3) of Poonchi bird showing different layers, koilin (K), mucosa (M), lamina propria submucosa (S), tunica muscularis (ML) and tunica serosa (Se). H&E, 40x.*

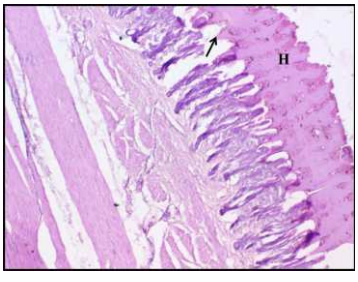

***Fig. 3** Photomicrograph of gizzard (G3) of Poonchi bird showing horizontal (H) and vertical (arrow) form of koilin. H&E, 100x*

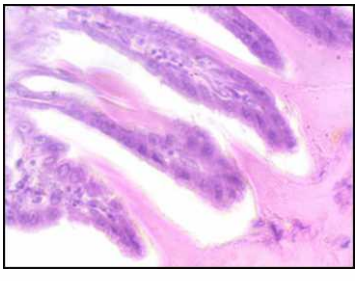

***Fig. 4.** Photomicrograph of gizzard (G1) of Poonchi bird showing simple columnar epithelium of mucosal folds. H&E, 1000x*

***Fig. 5.** Photomicrograph of gizzard of Poonchi bird showing glands (G) in lamina propria having collagen (red colour) and elastic (black colour) fibers. Von Geison & Verhoeff's stain, 400x*

***Fig. 6** Photomicrograph of gizzard (G3) showing presence reticular fibers. Gomori stain 400x*

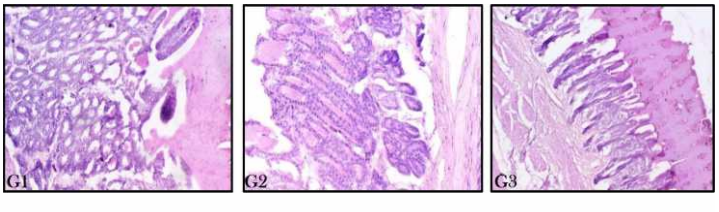

***Fig. 7.** Photomicrograph of gizzard of Poonchi bird showing simple tubular branched glands in G1 and G2 and simple tubular glands in G3. Glands in G2 showing prominent eosinophilic secretions as compared to G1 and G3. H&E stain, 400x*

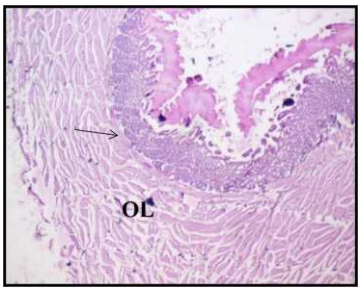

Fig. 8a. *Photomicrograph of gizzard showing presence of inner circular (arrow) and outer thick longitudinal muscle (OL) at G. H&E stain, 400x*

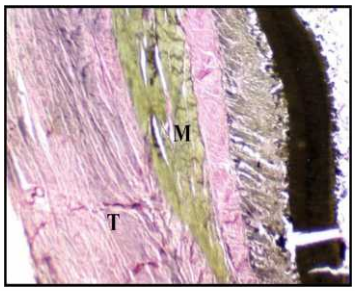

Fig. 8b. *Photomicrograph of gizzard showing presence of single layer of circular muscle (M) surrounded by thick collagenous layer (T). Von Geison & Verhoeff's stain, 400x*

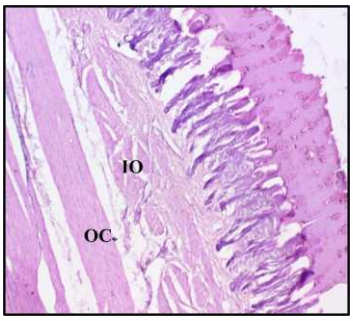

Fig. 8c. *Photomicrograph of gizzard showing presence of inner oblique (IO) and outer circular layer (OC) at G3. H&E stain, 100x*

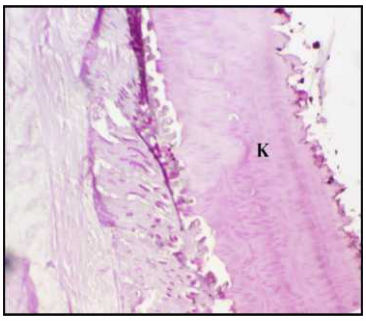

Fig. 9. *Photomicrograph of gizzard (G2) showing positive reaction of koilin (K) to PAS stain. PAS stain, 100x*

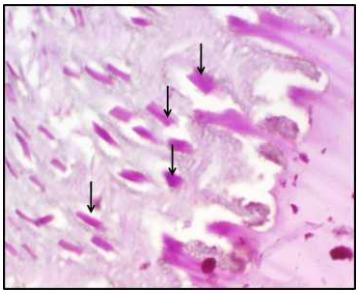

Fig. 10. *Photomicrograph of gizzard (G2) showing positive reaction of secretory material within the lumen of glandular tubules (arrow) to PAS stain. PAS stain, 400x*

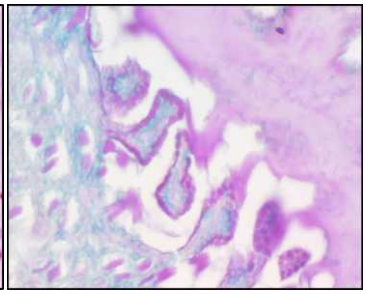

Fig. 11. *Photomicrograph of gizzard (G1) showing positive reaction of koilin (K) to PAS, mucosal epithelium of mucosal folds showing strong positive reaction for both PAS and AB. PAS-AB stain, 400x*

Fig. 12. *Photomicrograph of gizzard (G2) showing positive reaction of basic proteins in koilin (K) and tunica muscularis (M) Bromphenol Blue stain, 40x*

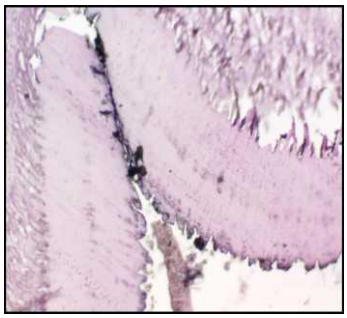

Fig. 13. *Photomicrograph of gizzard (G2) showing reaction of koilin (K) to sudanophilic lipids. Sudan Black B stain, 100x*

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