Histochemical and immunohistochemical investigations on pyloric tonsil in Turkeys (*Meleagris gallopavo*)

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ABSTRACT

The structural patterns of pyloric tonsil in turkey (*Meleagris gallopavo*) were investigated in this study. Turkeys, less resistant to disease than chickens, are a crucial source of protein with low cholesterol levels and a high protein/calorie ratio. Pyloric tonsil, involved in the chicken defense barrier, is a component of GALT. To evaluate the specific region, adult turkeys were taken from slaughterhouses. Crossman's modified triple staining demonstrated the general histological structure, PAS revealed the density of goblet cells synthesizing mucus, and Safranin O showed the capsule around the follicles. The width of lymphoid follicles in the pyloric tonsil (204.0±22.33) differed significantly from that of the middle part of the duodenum (111.7±4.741). It was noticed that the specific area harbors T cells, B cells, and follicular dendritic cells. Interfollicular regions were infiltrated with $CD3^{+}$T cells, but the $CD268^{+}$B cells occupied primarily germinal centers. However, it was noticed that T cells were present in germinal centers and some B cells in the interfollicular region. Anti-Vimentin antibody revealed follicular dendritic cells in the pyloric zone. It was noted that the epithelial layer of the pyloric tonsil is devoid of CK18-positive cells. Findings of the semi-thin section indicated that some columnar epithelial cells were stained differently in the transition zone (lymphoepithelium). To sum up, a pyloric region in turkeys is structurally dissimilar to pyloric tonsils detected in chicken, chukar partridge, and duck. More research is needed to reveal the effect of pyloric tonsil on the alimentary tract in poultry in terms of defense mechanisms.

Introduction

The immune system acts as a defensive tool for preventing or limiting infections. It has been suggested that the genes regulating the immune system have made notable evolutionary progress to block pathogens. In this regard, the immune surveillance system can recognize effectively and eliminate pathogens. However, pathogens continue to evolve new tactics to evade the immune system (25). In response to pathogens escaping defending mechanisms, the host increases its resistance over time. This well-established co-evolutionary dynamic has been postulated as the "host-pathogen arms race (6).

The poultry immune system has provided a crucial model for basic immunological studies. Chickens (*Gallus gallus domesticus*) have been included in most immunological investigations. With the seminal findings of avian immunology studies, basic immunological concepts are better understood. Undoubtedly, the remarkable contribution of the studies is to reveal in detail the development of humoral and cellular (cellular) responses, which are the two main branches of acquired immunity. The identification of developing T and B cell-dependent lymphocyte lines has been achieved smoothly. Furthermore, poultry species have contributed to the development of vaccine science (5).

Avian immunity depends on the function of lymphoid organs categorized as primary and secondary. Primary lymphoid organs such as bursa Fabricius and thymus are immune-privileged areas for antigen-independent differentiation and lymphocyte proliferation. Spleen, harderian gland, CALT (Conjunctiva-associated lymphoid tissue), NALT (Nasal region-associated lymphoid tissue), BALT (Bronch-associated lymphoid tissue), and GALT (Gut-associated lymphoid tissue) are

classified as secondary lymphoid organs and are the hub of adaptive immune responses (18). Studies have shown that GALT plays a key role in inducing local immune responses and protecting against mucosal pathogens (15). GALT contains pharyngeal tonsils, esophageal tonsils, Peyer's patches, caecal tonsils, and Meckel's diverticulum (18). In addition to these lymphoid structures, proventricular lymphoid tissue is involved in the poultry defense system (22).

The pyloric tonsil was first defined at the initial segment of the duodenum in a study performed on chickens. It has been reported that Lieberkühn crypts in this tonsillar area transformed into tonsillar invaginations, and the surface of the cryptic zone is covered with lymphoepithelium. Moreover, it was clarified that the localization of the lymphoid tissue of the pyloric region, unlike Peyer's patches on the antimesenteric side of the intestine, lies the entire wall of the intestine, thus forming a complete ring. In addition, it has been determined that the pyloric region contains at least 15-20 tonsillar units (16). Similarly, Gedam et al. (10) and Arugh and Hamedi (1) detected pyloric tonsillar units in ducks and chuckar partridges, respectively. It has been stated that tonsillar focal units were located in the lamina propria, especially adjacent to the muscularis mucosa layer. In addition, the capsular network surrounding each lymphoid structure fused with the connective tissue of the lamina muscularis layer (16). It has been declared that these tonsillar units were encountered in the submucosa and even in the lamina muscularis layer of ducks (10).

It is assumed that this lymphoid tissue has two pivotal functions. Firstly, the tonsillar region is an essential lymphoid structure and is heavily exposed to environmental antigens. Secondly, tonsillar areas may participate in the development of B-lymphocytes occurring mainly in Peyer's patches (18). Oláh et al. (17) stated that the tonsils function as a "gateway" for antigens. They stressed that an influx of antigens or allergens in the pyloric tonsil constantly stimulates the immune system and keeps it active.

This study, which was a determined road map based on previous studies revealing general features of the pyloric tonsil of avian species, aimed to define the structural characteristic of the pyloric tonsil in turkeys. It was planned to perform histochemical and immunohistochemical examinations to understand that this region is organized lymphoid tissue rather than lymphocyte infiltration.

Materials and Methods

Sampling: Both sexes of American bronze turkeys were utilized in this study [male: five adults (10-12 kg); female: five adults (8-9 kg)]. Ankara University Animal Experiments Local Ethics Committee approved this descriptive study (Ethics committee approval date: 15.03.2017, Decision number: 2017-6-50). The pyloric tonsilar region, located at the beginning of the duodenum, was taken from a total of 10 animals slaughtered in several slaughter houses around Ankara province. Both routine histological and electron microscopic fixation protocols were applied to tissue materials to reveal general structural configuration.

Preparation of samples for paraffin embedding and histochemical staining: The tissues were fixated with neutral buffered formalin (NBF) for 24 hours to detect normal histological structure. After the fixation process, they were exposed gradually to alcohol series (70%, 80%, 96%, and 100%) and immersed in methyl benzoate and benzol series. Following routine tissue processing steps, tissues were embedded in paraffin wax (2). Tissue blocks were sectioned by using the microtome (Leica RM2125 RTS). Afterward, 5-6 µm sections were kept in an oven (Core EN 500P) at 37 °C for 24 hours. Before staining paraffin sections, they were cleaned in xylene and rehydrated by immersing in serial dilutions of ethyl alcohol and water mixture. In the subsequent step, Crossman's modified triple staining method, PAS (Periodic acid-Schiff), and Safranin O staining methods were applied to different sections (2). Finally, they were photographed with a light microscope (Leica DM2500).

Immunohistochemical staining: To evaluate the pyloric tonsillar area immunohistochemically, tissue samples were firstly fixated with 10% NBF solution. Routine histological preparation protocol was conducted on samples, followed by tissue embedding step. After sectioning, serial paraffin sections (5µm thickness) mounted on poly-lysine-coated slides were used for immunohistochemical investigations. They were dried in the oven at 37 °C for 24 hours. Afterward, the sections were put in the oven at +58 °C for 30 to thaw parafin wax. After deparaffinization with xylene and alcohol series, they were washed in PBS for 5 min twice. Next, antigen retrieval was performed in 0.01 M citrate buffer (pH 6) at 100 °C for 20 min and then cooled at room temperature. 3% hydrogen peroxide solution were applied to sections for 20 minutes to prevent the activation of endogenous peroxides. Antibodies were diluated at a ratio of 1:50 for Anti-CD79A, Anti-Vimentin, and Anti-cytokeratin18 (CK18), 1:200 for Anti-CD268, and 10 µg/ml for Anti-CD3ϵ (Table 1). Prior to treated with primary antibody, serum blocking (Ultra V Block, TA-060-UB, Thermo Fisher Scientific) was performed in a humid chamber for 10 min. Following blocking nonspecific binding, antibody incubations were applied overnight at +4 °C and PBS were dropped on the negative controls. They were then incubated with biotinylated Goat Anti-Polyvalent secondary antibody (TP-060-BN, Thermo Fisher

Scientific) at room temperature for 30 min. Biotin-specific HRP (Horseradish Peroxidase) with Streptavidin was treated for 30 minutes. After this step, AEC (3-amino-9-ethyl carbazole) chromogen was applied to the sections in the dark for 1-5 minutes, and 3,3'-Diaminobenzidine (DAB) chromogen was performed on the section to which Anti-CD268 primer antibody was dropped only. Cell nuclei were stained with Gill's hematoxylin for 5 min. Subsequently, samples were coated with coverslips. Finally, images were captured under a light microscope (Leica DM2500).

Semi-thin sections: Additional tissue samples collected from a male and a female animal were prepared for the ultrastructural examination. For this purpose, tissues were immersed in glutaraldehyde-paraformaldehyde solution for 2-6 h. Washing step was carried out with 0.1 M Cacodylate buffer (pH 7.4) for 3 h. They were post-fixed in 1% osmic acid at 2 h. Removing osmic acid was achieved using cacodylate buffer for 20 min. The tissues were soaked in 30, 50, and 70% alcohol for 30 min, respectively. After immersing into uranyl dye, they were kept at +4°C for 2 h. Before embedding with Araldite, the tissues were rinsed with 96, 100, and propylene oxide for 1 h. Serial sections (1 µ thickness) were obtained by using a pyramitome, and then dyed with Toluidine blue. The sections were closed and photographed using a Leica DM2500 brand-fluorescent filter attachment microscope. Light micrograph images were acquired with the help of Leica DM2500 microscope.

Statistical Analysis: Statistical analyses were performed using GraphPad Prism (Version 5.00) software. Data (n=4) were given as mean ± standard error (SE). The diameter of lymphoid follicles was analyzed with the nonparametric Mann-Whitney U test to evaluate differences between the initial (pyloric region) and middle areas of the duodenum. Statistical significance was interpreted according to $P<0.05$ level.

Results

Triple staining: In this study, it was observed that a transitional region defined as the pyloric tonsil and localized at pyloroduodenal junction was filled with tonsillar units in turkeys. It was detected that lymphoid follicles and lymphoepithelium were primary components of the pyloric tonsil present at the initial segment of the duodenum. Lymphoid follicles in the tonsillar region of turkeys were scattered along the entire junctional wall rather than in the upper or lower part of the intestine, thus forming a complete ring. Lymphoid follicles (Width: 204.0±22.33) were located in the villi rather than in Lieberkühn crypts. It was noticed that lamina propria of villi contained 1-2 or more lymphoid follicles (3-5) of variable diameter. Additionally, the villi tended to expand in different directions, depending on the number and diameter of the follicles. It was noted that the number of follicles has a spatial relationship with the localization of lymphoid tissue. In this context, 1-2 follicles occupied the middle part of a villus, while 3-5 follicles were distributed from the bottom to the tip of a villus. This was a crucial indicator for the villus to demonstrate a capability of compensating for configurational alterations. However, some villi are devoid of lymphoid follicles (Figure 1. A-C). The epithelial transformation was another finding of this study. The epithelial cell layer lining the villi containing the lymphoid follicle exhibited a gradual alteration from columnar to squamous. However, this lymphoepithelial change did not occur along the entire length of the villi, the lymphoepithelium was detected commonly at contact sites where an epithelial layer of a villus fused with an epithelial layer of another villus. Finally, it was determined that goblet cells were in this area (Figure 1. D).

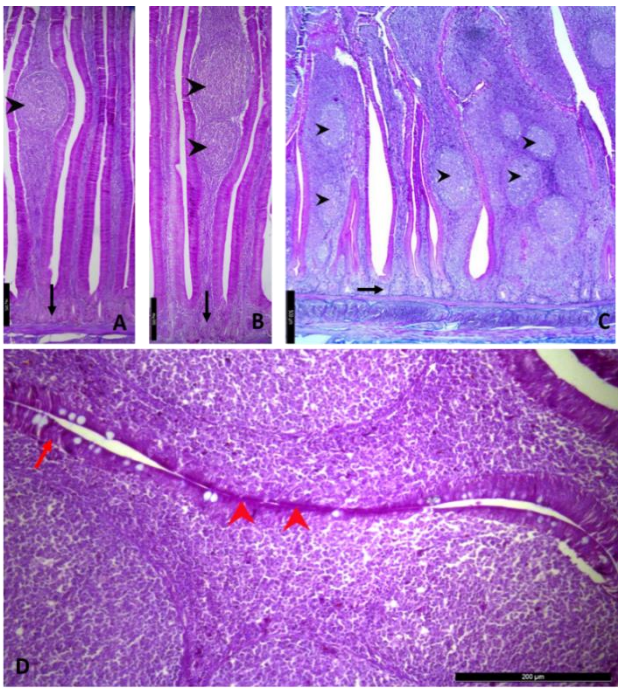

Figure 1. General view of the pyloric tonsil area.
A-B: Lymphoid follicles in villi, X100. C: Villi of different diameters in the pyloric tonsil area, X50. D: Lymphoepithelium, 200X. Staining: Crossmann's modified triple. Black arrow: Lieberkühn crypt. Black arrowhead: Lymphoid follicle. Red arrow: Simple columnar epithelium. Red arrowhead: Lymphoepithelium.

Safranin O and PAS staining: To visualize a capsular network around the germinal center and yield reliable results, the Safranin O staining procedure was applied on sections. The presence of a capsule around the germinal center was detected. It was revealed that the capsule reacting positively to Safranin O was stained pink (Figure

2. A). After the junction area, it was noticed that the lymphoid follicles (Width: 111.7±4.741) in the villi decreased both numerically and volumetrically in Figure 2-B. Width of lymphoid follicles in middle portion of duodenum showed statistical difference ($P<0.05$) with that of pyloric tonsillar follicles (Figure 3). The villi were devoid of fusion sites any longer. Another result of the PAS staining method was the detection of goblet cells in the villous epithelium covering lymphoid follicles. It was determined that the capsule, which reacted positively with safranin O, was also stained with PAS (Figure 2. C).

Immunohistochemical staining: Results obtained by immunohistochemical stainings are summarized in Table 1. Detection of T cells in the pyloric tonsil region was achieved using an Anti CD3ϵ antibody. From a general perspective, lymphoid follicles are localized along the pyloric region of the digestive tract to form a ring-shaped structure. This finding was compatible with the triple result. Black arrows represented the interfollicular area and black arrowheads demonstrated positive T cells in the germinal center in Figure 4. CD3+ T cells populated widely in the interfollicular area, but small quantities of T cells were also determined in germinal centers (Figure 4. A-B). Apart from this, T lymphocytes (red arrowhead) were detected in the intraepithelial area (Figure 4. A).

Two different clones were exerted to show B lymphocytes in the region. Anti-CD268 BAFF-R (MCA6011GA) was conjugated with CD-268, thereby giving a positive reaction. Conversely, the Anti CD79a-HM57 (NB100-64347) clone failed to bind to the cell surface antigen. Unlike T lymphocytes expressing CD3, B lymphocytes expressing CD268 antigen were primarily observed in the germinal center of the follicle (black arrow). They were also present in the interfollicular areas where T cells were abundant. In other words, $CD268^{+}$ B cells were noticed both in the follicle and the interfollicular area (Figure 5. A-C).

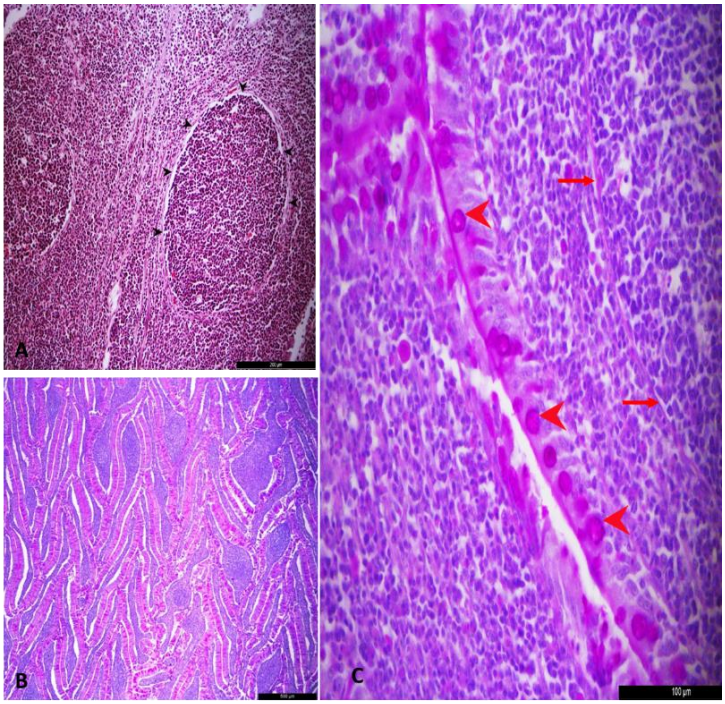

Figure 2. A: Image of a capsular structure around a follicle, X200. Staining: Safranin O. Black arrowhead: Capsule. B: The general structural patterns of the villi in the area slightly ahead of the pyloric tonsil region and the showing of considerably shrunken lymph follicles in some villi by PAS staining, X50. C: Presence of capsule and distribution of goblet cells in pyloric tonsil, X400. Staining: PAS. Red arrow: Capsule. Red arrowhead: Goblet cell.

Table 1. Antibody types, dilutions, and immunohistochemical staining results.

Antibody	Type	Clone	Dilution	Result
Anti-CD3ϵ	Polyclonal	CD3 A0452 Dako	10 µg/ml	Positive
Anti-CD79a	Monoclonal	Anti CD79A-HM57 (NB100-64347)	1:50	Negative
Anti-CD268	Monoclonal	Anti CD268 BAFF-R (MCA6011GA)	1:200	Positive
Anti-Vimentin (RV202)	Monoclonal	OMA 1-06001	1:50	Positive
Anti-CK18	Monoclonal	Anti Cytokeratin18 (NBP1-97715)	1:50	Negative

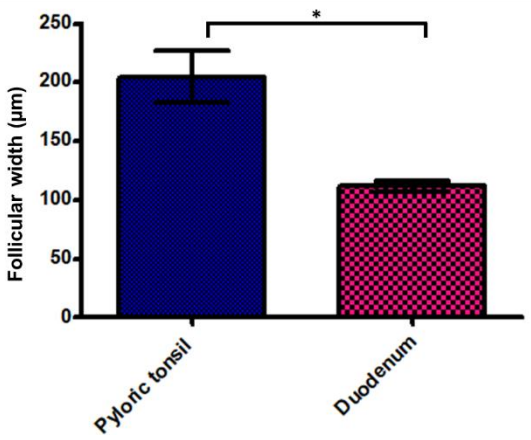

Figure 3. The width of lymphoid follicles measured in pyloric area and midportion of duodenum.
* P<0.05. Mann-Whitney U test was used.

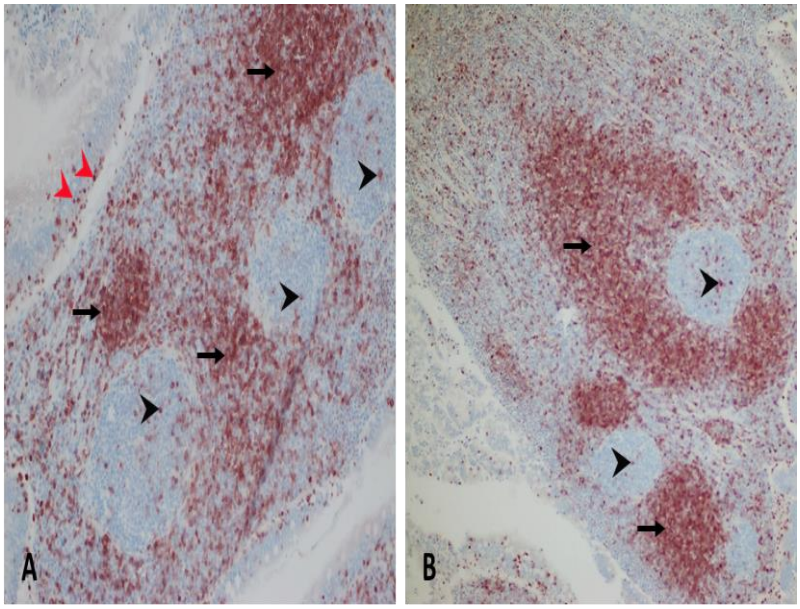

Figure 4. Demonstration of positive reaction in different regions and at different magnifications.
A: Detection of $CD3^+$ T cells in interfollicular region (black arrow) germinal center (black arrowhead) and epithelium (red arrowhead), 100X. B: Manifestation of $CD3^+$ T cells in interfollicular region (black arrow) and germinal center (black arrowhead), 200X.

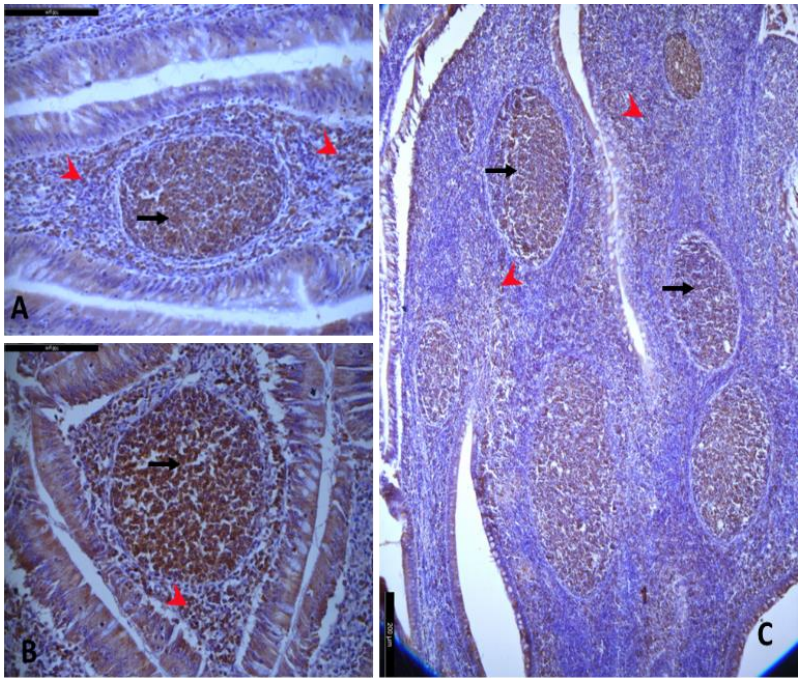

Figure 5. Detection of positive reactions in three different areas and at different magnifications.
A-B: Localization of CD268+ B cells in germinal center (black arrow) and interfollicular area (red arrowhead), X400. C: CD268+ B cells in germinal center (black arrow) and interfollicular area (red arrowhead), X100.

To identify follicular dendritic cells in the pyloric tonsil, serial sections were incubated with Anti-Vimentin antibody. As a result of immunohistochemical staining, it was noted that follicular dendritic cells (black arrows), an antigen-presenting cell in the follicles, were stained positively. Similarly, positive reactions were observed in some other cells (black arrowheads) in the outer part of the follicle (Figure 6). The reason why those cells showed positive reactions may be that vimentin is one of the basic elements of the cytoskeleton.

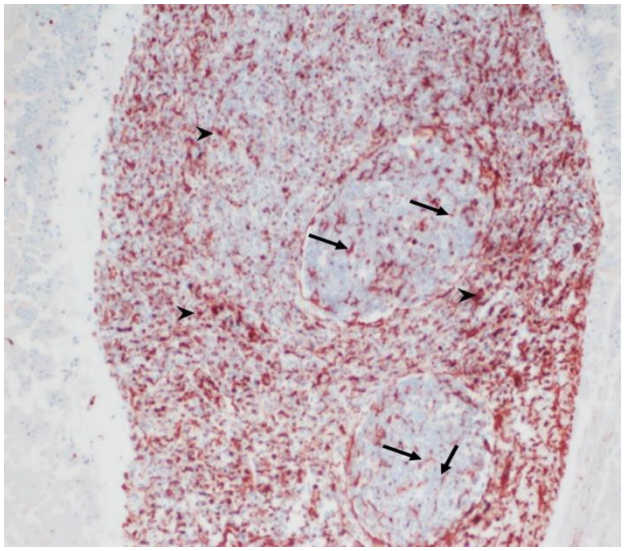

Figure 6. Localization of follicular dendritic cells in the pyloric tonsil, X200.
Black arrow: Follicular dendritic cells. Black arrowhead: Other cells expressing Vimentin.

The main purpose of using the anti-CK 18 antibodies was to reveal the localization of M cells in the lymphoepithelium. However, no cells expressing cytokeratin 18 were detected in the epithelial layer. This result indicated that M cells in the pyloric tonsil could not synthesize the intermediate protein.

Semi-thin sections: The presence of lymphoepithelial transformation in semi-thin sections stained with toluidine blue was detected at various magnifications (200X, 400X, and 1000X). Both the preparatory phase and the places where the transformation from the columnar epithelium to the lymphoepithelium took place were determined in the same area (200X). A group of blue-stained prismatic cells, which were the same size as the normal single-layered columnar epithelium and were thought to be preparing to transform into lymphoepithelium, were observed (Figure 7. B). The color of these epithelial cells changed from white to blue. It was assumed to be the case that cells first altered their functional character and then shortened their length. The localization of squamous epithelial cells, which we previously found out with triple staining (Figure 1), was revealed in more detail with 1µ sections. It was noticed that lymphocytes migrating inside lymphoepithelium were packed in the epithelium. The number of these lymphocytic cells (black arrowheads) reached roughly 5-6 (Figure 7. C). It has been concluded that the epithelial cells associated with lymphoid follicles may function the way packaging lymphocytes similar to M cells.

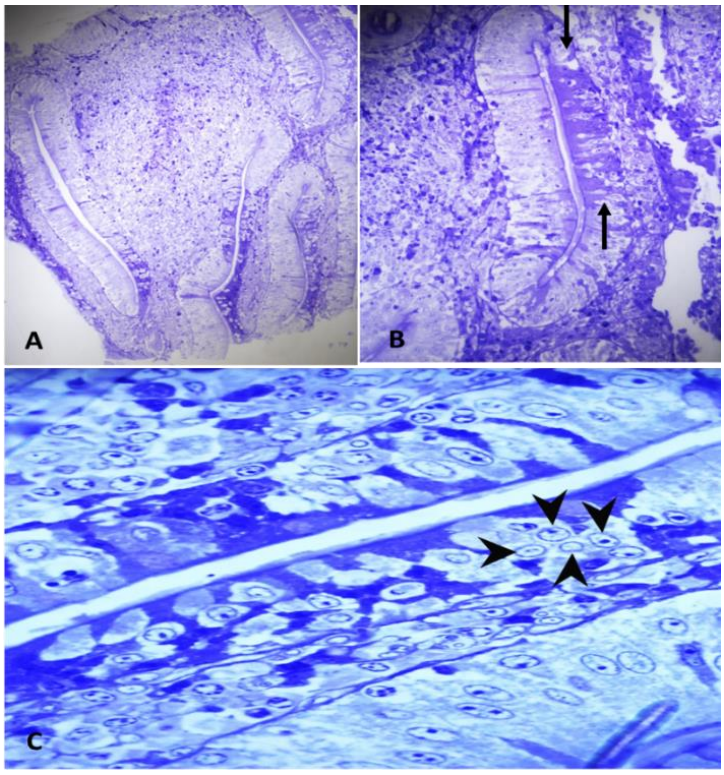

Figure 7. Demonstration of lymphoepithelium in serial semi-thin sections (Staining: Toluidine Blue).
A: Detection of general structural features, 200X. B: Presence of a group of blue-stained prismatic cells, 400X. Black arrow: Epithelial area occupied by blue-stained prismatic cells. C: Manifestation of lymphocytes packed by epithelial cells, 1000X. Black arrowhead: Intraepithelial lymphocytes.

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Discussion and Conclusion

The pyloric tonsil, absent from mammalian species, is located in the entrance region of the duodenum and was first determined in chickens. Researchers suggested that the pyloric tonsil takes part in fighting against pathogens (4, 16). After revealing this structure in chickens, it has been a matter of curiosity whether this tonsillar area is similar in other poultry. For this purpose, similar studies were carried out in ducks (10) and chukar partridges (1), and the presence of the pyloric tonsil in these poultry species was detected. No study has been found in the literature on the histological structure of this region in turkeys. In the present study, the histological structure of the part referred to as the pyloric tonsil region in turkeys was unveiled and discussed with literature data in this area. It has been stated that these tonsillar units consisting of 15-20 lymphoid follicles are localized in the intestine's upper and lower walls, while lymphoid follicles in the ileum and cecum are located in the anti-mesenteric region (16, 18). Our findings regarding the distribution of lymphoid tissue in turkeys were similar to the results of other studies.

It has been reported that the digestive and respiratory tracts contain tonsillar structures since they are exposed to the invasion of many foreign antigens (4). Tonsils are generally defined as lymphoid follicles located within the mucosal layer (7, 21). Perry (19) mentioned crypts ending with many blind ends in the tonsillar region, their lateral branches, and lymphoid tissues in these parts. However, Oláh et al. (17) described the tonsil as a complex structure containing a depression called a crypt surrounded by dense lymphoid tissue. Casteleyn et al. (4), conducted on esophageal, proventricular, pyloric, and caecal tonsils, stated that lymphoid tissues were all observed at the cryptic regions and close to muscular mucosae. Considering the results of this study, the localization of lymphoid tissue in the pyloric tonsil region was different. In this context, it was determined that the villi and crypts in the pyloric tonsil region of turkeys were similar to a normal intestinal structure. The lymphoid follicles were mostly located at different levels within the villi rather than the crypts. Furthermore, it was revealed that these lymphoid follicles give rise to a volumetric increase and shape alteration in villi. All the dissimilarities mentioned above have been one of the main differences between our findings and other studies.

On the one hand, Nagy and Oláh (16) stated that Lieberkühn crypts transformed into lymphoepithelial tonsillar crypts and that there were primary and secondary lymphoid follicles and interfollicular regions in the lamina propria. On the other hand, Gedam et al. (10) stated that there are lymphoid follicles in the submucosa and even in the muscularis mucosa. In this study, lymph follicles were not detected along the crypts. Moreover, no lymphoid follicles were observed in the muscularis mucosa or the submucosa. It has been revealed that there is a positive relationship between the number of lymph follicles and their localization. Accordingly, it was determined that lymphoid follicles (1-2) were commonly located in the middle part of a villus and had a different localization (from the base to the tip) into a villus, depending on the number of follicles. Apart from this, the width of lymphoid follicles in the pyloric tonsil (204.0±22.33) differed significantly from that of the middle part of the duodenum (111.7±4.741). The reason why there are larger follicles in the pyloric tonsil may be that it may be exposed to a high microbial load. Unlike other studies (1, 4, 10, 16) 5-6 lymph follicles were observed in a villus. In addition, it has been demonstrated that the villi can expand depending on the localization of the lymph follicles.

Nagy and Oláh (16) reported that most of the circular-shaped germinal centers of lymph follicles are located close to the muscularis mucosa of the intestine. They stressed that the capsule, which consists of type III collagen around these follicles, fuses with the connective tissue capsule of the muscularis mucosa layer. Nonetheless, no fusion has been noted based on the difference in the location of the lymphoid follicles in the present study. On the other hand, the capsule surrounding one germinal center did not merge with that of another germinal center in conjunction with the findings of other researchers (10, 16).

Mason et al. (14) stated that B cells, including mammalian progenitor B cells, express the CD79a antigen. Furthermore, the expression of CD79a antigen on the surface of B cells was detected at all stages of the differentiation process involving plasma cells. However, it was revealed that B-lymphocytes in the turkey pyloric tonsil did not synthesize this receptor in the study. To identify B cells in the region, CD268 antigen expression of peripheral B cells (20) was demonstrated immunohistochemically. B cells were densely located in germinal centers. The presence of positive-reacting B cells in the interfollicular areas, the T-lymphocyte region, revealed similarity with the BALT tissue study (8). These lymphocytes were also found in the intraepithelial area. Oláh et al. (18) stated that T cells accumulate T cell-dependent regions, interfollicular areas, in the caecal tonsils and Peyer's plaques. Similarly, Nagy and Oláh (16) showed that T cells densely located in interfollicular regions stained positively with anti-CD3 antibodies. Apart from this, they revealed that T cells were also present substantially in the germinal center. The presence and localization of CD3+ T cells in turkeys were similar to other studies.

It has been reported that heterogeneous intraepithelial lymphocytes settle between epithelial cells in the mucosal layer along the gastrointestinal tract (9).

Generally, T cells inhabit the epithelial layer, while B-lymphocytes mostly remain in the lamina propria (11). Vervelde and Jeurissen (24) noticed that the stratified squamous epithelium of the esophagus and the simple columnar epithelium of the intestines harbor lymphocytes. CD3+ intraepithelial lymphocytes are predominantly CD8a+ cells, most of which express TCRγδ (3). In the current study, localization of CD3+ T-lymphocytes in the epithelial layer was also observed in turkeys.

Follicular dendritic cells (FDCs), a rare type of stromal cell, resides in follicles of secondary lymphoid tissues. FDC, which defines the light zone of the GC, is crucial for GC formation and maintenance. They bind and store antigens in the form of immune complexes (ICs) for presentation to GC B cells (23). In the study revealing the pyloric tonsil region for the first time in chickens (16), the presence of vimentin intermediate filament in follicular dendritic cells was indicated. Follicular dendritic cells expressing vimentin were also detected within the germinal centers in the present study.

Nagy and Oláh (16), conducted on chickens, stated that a capsule encircled germinal centers of lymph follicles in the pyloric tonsil region. Likewise, it was observed that the capsule surrounded lymph follicles of the pyloric area in turkeys. Hondo et al. (12) stated that they observed CK18-positive M cells in the follicle-associated epithelium of the jejunal and ileal region in cattle. As a result of examining semi-thin sections, some specialized epithelial cells packaging of a few lymphocytes were noticed in this study. That is why they were considered M-cell-like cells. However, the sections incubated with CK18 stained negatively.

While lymphoepithelium was observed along the crypt in the tonsils (17), it was reported that lymphoepithelial transformation was detected only dome region in the ileum (13). In this study, lymphoepithelium was observed in the contact site of the villi. It was revealed that the simple columnar epithelium gradually became the lymphoepithelium in semithin sections.

In conclusion, the pyloric tonsil, stated in the literature to be related to the defense mechanism, localized between the stomach and duodenum, structurally different from the duodenum and pylorus, has been revealed by using histological and immunohistochemical methods in turkeys. Contrary to other studies, it was observed that the pyloric tonsil region of turkeys was in a typical intestinal structure. Furthermore, it was determined that the lymphoid follicles, which are the main components of this region, were not located at the crypt as claimed in the literature. As a result of the examination, a remarkable finding was revealed that follicles developed in different numbers and sizes within the villi. Further studies, however, are required elaborately to demonstrate the defense mechanism of the digestive tract in poultry.

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Conflict of Interest

The author declared that there is no conflict of interest.

Author Contributions

NY conceived and planned the experiments, carried out the experiments, interpreted the results, and took the lead in writing the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

The author confirm that he has adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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