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Morphometric characteristics of the lymphatic nodules in the porcine gallbladder

Ivaylo Stefanov[1], Stefan Stefanov[3], Nikolay Tsandev[2], Ivelina Ivanova[1], Sevinch Hamza[1], Vanessa Pilicheva[1], Angel Vodenicharov[2]*

[1] *Department of Anatomy, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria*
[2] *Department of Veterinary Anatomy, Histology and Embryology, Trakia University, Stara Zagora, Bulgaria*
[3] *Fifth year student at Medical faculty, Trakia University, Stara Zagora, Bulgaria*

***** Corresponding author e-mail: ivstefanov@abv.bg

The aim of this study was to estimate the distribution and size of the lymphatic nodules in the wall of gallbladder in pigs at different ages.

A light microscopic observation was performed on the lymphatic tissue localized in *fundus*, *corpus* and *collum vesicae felleae*. We found out that lymphatic tissue in gallbladder is represented by diffuse lymphatic tissue, primary and secondary type of solitary lymphatic nodules as well as aggregated lymphatic nodules. In 2 month-old pigs, only diffuse lymphatic tissue was observed. In 6 month- and 3 year-old animals except for diffuse lymphatic tissue, both primary and secondary lymphatic nodules were defined. The density of nodules in 6 month-old pigs was higher than in 3 year-old ones. In 6 month-old pigs, the diameter of nodules was smaller than in 3 year-old ones.

In conclusion, the solitary and aggregated lymphatic nodules observed form well defined gallbladder-associated lymphoid tissue.

Key words: gallbladder – associated lymphoid tissue, morphometry, pigs

Introduction

Based on the anatomical and functional properties, the mucosal immune system (MIS) can be separated into inductive and effector sites [9]. The migration of immune cells from mucosal inductive to effector tissues via the lymphatic system is the cellular basis for the immune response in the gastrointestinal, the upper respiratory, and female reproductive tracts. Mucosal inductive sites include mucosa-associated lymphoid tissue (MALT) network delivering a continuous source of memory B- and T-lymphocytes that then move to mucosal effector sites [5, 7]. Mucosal effector sites, including the lamina propria regions of the gastrointestinal, the upper respiratory and female reproductive tracts as well as secretory glandular tissues (mammary, lacrimal, salivary, etc.) contain

Ag-specific mucosal effector cells such as IgA-producing plasma cells, and memory B and T cells [1]. The components of MALT include solitary lymphoid nodules, aggregates consisting of several lymphoid nodules and lymphoepithelium (LE) [8].

The uptake of antigen occurs through the lymphoepithelium (LE) by M cells or dendritic cells. Some components of MALT are constitutively present at defined mucosal sites, such as the tonsils; others are with varying location, such as Peyer's patches in the jejunum and ileum, and others are induced by antigen exposure, such as bronchus-associated lymphoid tissue and solitary lymphatic nodules. Recirculating lymphocytes enter MALT through venules in the internodular areas. Receptors on venules regulate the tissue-specific migration of lymphocytes [8].

The structure of the lymphoid tissue in porcine stomach, small and large intestine is well studied [6, 15]. Recently, Stefanov [14] studied the age-dependent distribution and size of lymphatic nodules as components of extrahepatic bile duct associated lymphatic tissue in domestic swine. However, information about the existence and structure of the lymphoid tissue in gallbladder in domestic swine (even in Nomina Histologica Veterinaria [12]) is not available.

That`s why the aim of the current study is to define the distribution and size of lymphatic nodules (LNs) in the wall of gallbladder in pigs at different ages in order to elucidate the structure of gallbladder-associated lymphoid tissue (GBALT) as another component of mucosa-associated lymphoid tissue.

Materials and Methods

Animals

For this study, gallbladders of 6 pigs at the age of 2 months (22 – 33 kg), 6 pigs at the age of 6 months (92 - 100 kg) and 6 pigs at the age of 3 years (280 - 300 kg) were collected according to the Scientific Project number 13/2017, Medical Faculty, Trakia University, Stara Zagora, Bulgaria. All procedures were carried out in accordance with the Bulgarian legislation regarding animal care (Ordinance 20 of 01.11.2012 on the minimum requirements for the protection and welfare of experi mental animals and the requirements for the sites for use, breeding and/or delivery) and Directive 2010/63 / EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

Material

The tissue samples were obtained from pigs slaughtered at slaughter house for meat consumption. The samples from gallbladder`s bottom, body and neck were fixed in 10% aqueous solution of formalin. Then these samples were dehydrated in alcohols and embedded in paraffin. About 5-6 μm thick paraffin serial cross sections were mounted on gelatinized slides, twice placed in xylene, rehydrated by decreasing ethanol concentrations and stained with Haemotoxylin and Eosin (H&E) dye.

Histochemical methods for detection of elastic and collagen fibers

Orcein staining method was used to identify elastic fibers (stained in dark brown) but Van Gieson method – for detection of collagen fibers (stained in red).

Morphometric study

The measurements were performed on sections stained with H&E in order to define the size (diameter, height and width in μm) and density (number per cross section) of LNs. The measurements were done by a light microscope (LEICA DM1000, Leica Microsystems, UK) with a digital camera (LEICA DFC 290) and software (LAS V4.10.0 2016).

Statistical analysis

The data obtained were processed by GraphPad Prism 6 for Windows (GraphPad Software, Inc., USA) via one-way analyses of variance (one-way ANOVA) followed by Tukey-Kramer's post-hoc test. The values of the studied parameters were presented as mean ± SD. P-values < 0.05 were considered statistically significant.

The terminology was consistent with the Nomina Histologica Veterinaria [12].

Results

In the present study, a light microscopic observation was performed on the lymphatic tissue localized in the three parts of the gallbladder: bottom (*fundus vesicae felleae*), body (*corpus vesicae felleae*) and neck (*collum vesicae felleae*).

It was found that the gallbladder-associated lymphatic tissue was represented by diffuse lymphatic tissue (DLT), solitary lymphatic nodules (SLNs) and aggregated lymphatic nodules (ALNs). SLNs and ALNs were absent in 2 month-old pigs but were present in 3 year – and 6 month-old animals. The micromorphometric study allowed calculating the diameter, height and width of SLNs and ALNs (**Table 1**).

In **2 month-old pigs,** DLT is the main lymphoid tissue localized in the propria (*lamina propria mucosae*) below the surface simple columnar epithelium (*lamina epthelialis mucosae)*, but SLNs were not observed. The DLT in the propria of the *fundus* of gallbladder was less developed and looser than in the gallbladder`s body. In the neck of gallbladder, the DLT was most developed and extends into muscle layer (*tunica muscularis*) of the organ where surrounds the gallbladder`s glands (*gll. vesicae felleae)* (**Fig. 1a,b**).

In **6 month-old pigs**, loose DLT was present in the propria of *fundus* filling the connective tissue of the mucosal folds. This group of pigs, unlike 2 month-old pigs, showed well developed SLNs arranged in single row in the propria protruded to the lumen of bladder. These protrusions, covered with unfolded mucosa, were situated between mucosal folds of gallbladder`s *corpus* and *collum*. The peripheral part of the LNs penetrates the inner part of the muscle layer at the border with the propria. In most of SLN, *corona lymphonoduli* and germinal center were not defined. Single LNs with distinctive *corona lymphonoduli* and germinal center were observed (**Fig.1c**). Above nodules, between them and *lamina epithelialis mucosae,* a low and wide area of lymphocytes, known as dome, was determined. The DLT in *corpus vesicae felleae* was more developed and denser than those in the *fundus* and was localized in the remaining parts of the propria where LNs were not present. Except of the propria,

the DLT was also observed in the muscle layer surrounding the glandular alveoli. In the *collum vesicae felleae,* the DLT and SLNs were localized in the same manner like in gallbladder`s body. However, single (1-2 nodules per cross section) pear-or oval shaped lymphatic nodules were localized deeply in *tunica muscularis*. The SLNs were not surrounded by connective tissue capsule except of those localized in muscle layer which were surrounded by delicate capsule. The basal part of each nodule was separated from underlying muscle layer by delicate connective tissue area containing collagen and elastic fibers, as well as vessels of the microcirculatory bed: arterioles, capillaries and venules. The lymphoid tissue was represented mainly by primary and less secondary SLNs separated by internodular area containing lymphocytes (DLT), collagen and elastic fibers, as well as vessels of microcirculatory bed (**Figs. 1, 2**). The vessels were observed mostly in the basal part of nodules, as well as in domes at the border with peripheral part of nodules and between *corona lymphonodul i*and germinal center in secondary LNs.

In *corpus vesicae felleae*, ALNs, consisting of 3 LNs in most cases, were localized in the propria and protruding to the lumen of bladder between mucosal folds (**Fig. 1c**). In the surface epithelium above the ALNs single goblet cells were identified.

The ALNs in gallbladder formed nodular area, internodular area and nodule-associated epithelium (**Fig. 1c**).The internodular area was represented mainly by DLT and vessels of microcirculatory bed. The nodule-associated epithelium was observed to consist of simple columnar epithelium above the nodules and domes, lymphocytes as well as has single or no goblet cells.

Rarely, the base of ALNs together with gallbladder glands formed lymphoglandular complex (LGC) which was formed mostly by diffuse lymphatic tissue and gallbladder`s glands (**Fig. 1b**), but single lymphatic nodules were detected near the glands. LGC epithelium was represented by secretory glandular cells, goblet cells and lymphocytes. In *collum vesicae felleae*, ALNs, consisting of 2 LNs, were localized in the propria and protruding to the lumen of bladder between mucosal folds.

Single goblet cells (from 1 to 3 cells) were observed in the surface epithelium above the ALNs.The surface epithelium of the adjasent mucosa contained more goblet cells (**Fig. 1d).**

The diameter of ALNs in *collum vesicae felleae* was bigger than in *corpus vesicae felleae*. In 6 month-old pigs the diameter of ALNs was larger than in 3 year-old animals.

In **3 year-old animals**, the localization of solitary LNs and DLT was similar to 6 month-old pigs.

The main shape of nodules in gallbladder wall was round – their height and width were almost the same (**Table 1**). The diameter of SLNs was largest in the neck, followed by the body, and smallest in the bottom of gallbladder in both groups: of 6 month- and 3 year-old animals. The number of SLNs was highest in the body, followed by the bottom and smallest in the neck of gallbladder in 6 month- and 3 year-old animals. Most LNs showed raised areas called domes, which were observed as low and wide subepithelial regions in the gallbladder of 6 month-old pigs but were better defined in the three parts (bottom, body and neck) of gallbladder in 3 year-old animals. The surface epithelium over the dome region was devoid of folds or only single thin and short folds can be detected. This nodule-associated epithelium overlying the lymphatic

nodules consists of simple columnar epithelium with single goblet cells (**Fig. 1d**). The surface epithelium was infiltrated with lymphocytes (**Fig. 1b**).Therefore the nodule-associated epithelium is kind of lymphoepithelium. The basement membrane of the surface epithelium participating in formation of lymphoepithelium was more porous than that of the surface epithelium between nodule-containing areas.

In 3 year- old animals, ALNs, consisting of 2 lymphatic nodules, were localized in the propria and protruding to the lumen of gallbladder`s bottom, body and neck between mucosal folds (**Fig. 1d**). The diameter of ALNs in the neck was the biggest, followed by bottom and body of bladder. Like in 6 month-old pigs, the LGC was formed mainly by DLT around and between gallbladder`s glands, but single SLNs were detected near the glands. Lymphoglandular complex epithelium was observed to contain secretory glandular cells, goblet cells and lymphocytes

The number of SLNs in both 3 year- and 6 month-old animals was highest in *corpus*, followed by *fundus* and *collum vesicae felleae*. However, in 6 month-old animals the density of SLNs was significantly higher than in animals at the age of 3 years (**Table 2**).

Discussion

In the current light microscopic study, the detailed information about the structure of gallbladder-associated lymphatic tissue was delivered for the first time. It was revealed that GBALT was represented by DLT, solitary lymphatic nodules – *noduli lymphatici solitarii* [12] similarly to GALT in small and large intestine described by Urmila et al. [15]. With advancing age, the GBALT developed and primary and secondary LNs were seen in the gallbladder. Lymphatic tissue developed until six months. No signs for lymphatic tissue involution were seen at the age of six months in the GBALT. However, such involution was detected at the age of 3 years.

The results showed that the lymphoid tissue forming in the gallbladder of two-month-old pigs was represented by DLT only. The SLNs in gallbladder appeared in pigs at six months of age and were arranged in single row without germinal center, only single LNs showed germinal centers. This finding is similar to the localization of SLN in submucosa of porcine duodenum but is different from their location in jejunum and ileum, where LNs were arranged into two rows [15]. The lymphoid tissue gallbladder had primary and secondary LNs separated by internodular areas. This lymphoid tissue was seen in specimens taken from pigs older than two months.

The current study showed that the diameter of the LNs in gallbladder increased with age from fundus to *collum vesicae felleae* and was similar to diameter of LNs in porcine intestine [15]. Also, they were oval shaped resembling the SLNs in porcine duodenum [15].

The SLNs in gallbladder were not enclosed by connective tissue capsule except those localized in muscle layer which were surrounded by delicate capsule. The lymphoid tissue had mainly primary and less secondary LNs separated by internodular areas containing lymphocytes, vessels of microcirculatory bed and collagen- and elastic fibers. These results correspond to the findings of Urmila et al. [15] in porcine intestine.

As mentioned above, in porcine gallbladder both types of LNs were detected: primary and secondary ones. The secondary LNs showed pale germinal center and dense *corona lymphonoduli*. Similar to porcine intestine, over the LNs areas called domes were observed [15]. In gallbladder, the domes were low and broad. The domes of the jejunal and ileal lymphoid nodules were found to possess broad basal portion and a narrow apical part [15]. According to Neutra et al., [11] the dome contains B- and T-lymphocytes, dendritic cells (DCs) and macrophages. Morfitt and Pohlenz [10] reported that gastric MALT nodules resemble lymphoglandular complexes described in the colon of pigs. The gastric epithelium is devoid of parietal and goblet cells in these areas and releases deep crypts with areas of lymphoepithelium between the lymphoid follicles. Solitary LNs were observed in the submucosa and lamina propria of the lesser curvature of the gastric cardia and of the cardiac fundic diverticulum [6]. Later, in healthy pigs, some authors found that gastric MALT was present in fetal pigs and at birth like the other MALT structures of the gastrointestinal tract [3]. In piglets, they are described as inactive encapsulated aggregates of lymphocytes deep in the submucosa [6]. Green et al. [6] reported that activation of gastric MALT can be induced by colonization of piglets with Helicobacter pylori, but not by enteric bacteria or viruses.

The intestinal aggregated lymphatic tissue was described in details by Eurell and Frappier, [4]. Aggregated lymphatic tissue of the intestine contains submucosal lymphatic nodules with high mitotic activity, a zone of small lymphocytes (the *corona lymphonoduli*), internodular region rich in T cells and postcapillary venules through which lymphocytes recirculate, an elevated region (the dome) overlying LNs, and a nodule-associated epithelium. Like in rabbit intestine [11], the ALNs in gallbladder observed in this study form nodular area, internodular area and nodule-associated epithelium.

The LNs were observed near the gallbladder`s glands forming LGC. The current study showed that LGC epithelium in porcine gallbladder consisted of glandular secretory cells, goblet cells, intraepithelial leukocytes. The LGCs were present in the *corpus vesicae felleae* of 6 month-old pigs but not in 3 year-old animals. Taking into account the classification of LGCs in porcine intestine [15], we suggest that most LGCs in gallbladder belong to superficial type, because they were localized in *lamina propria mucosae.* Single LGCs were observed deep in the muscle layer of gallbladder`s *collum*.

The lymphoepithelium was described on jejunal Peaer`s patches, ileal Peaer`s patches domes and in LGC in the large intestine [2, 8, 10, 13]. It was reported to consist mostly of enteroabsorptive cells and single M-cells. In gallbladder, the lymphoepitelium, also called nodule-associated epithelium, was presented by simple columnar epithelium infiltrated by lymphocytes. M-cells of lymphoepitelium in gallbladder were not described because they were not the aim of the current study.

The structure of LGCs observed in gallbladder was similar to that of LGCs in porcine intestine described by Morfitt and Pohlenz [10]: LNs and internodular lymphatic tissue penetrated by gallbladder`s glands. In the intestine, the LGC epithelium contained goblet cells, enterocytes, enteroendocrine cells, M cells, individual and grouped intraepithelial leukocytes [10], but in gallbladder LGC epithelium consisted of secretory glandular cells, single goblet cells and intraepithelial lymphocytes.

Conclusion

The presence of lymphatic nodules and aggregates forming gallbladder-associated lymphoid tissue defines the gallbladder`s mucosa as important part of the mucosal immune system in pigs.

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References

1. Brandtzaeg, P. Induction of secretory immunity and memory at mucosal surfaces. – *Vaccine*, **25**, 2007, 5467-5484.

2. Chu, R. M., R. D. Glock, R. F. Rossand, D. E. Cox. Lymphoid tissues of the small intestine of swine from birth to one month of age. – *Am. J. Vet. Res.,* **40**(12), 1979, 1713-1719.

3. Driessen, A., C. Van Ginneken, J. Creemers, I. Lambrichts, A. Weyns, K. Geboes N. Ectors. Histological and immunohistochemical study of the lymphoid tissue in the normal stomach of the gnotobiotic pig. – *Virchows Arch*., **441,** 2002**,** 589-598.

4. Eurell, J. A., B. L. Frappier. Dellmann's textbook of veterinary histology, 6th ed. Blackwell Publishing Professional 2121 State Avenue, Ames, Iowa 50014, USA, 2006, pp. 139-143.

5. Fujihashi, K., P. N. Boyaka, J. R. McGhee. Host defenses at mucosal surfaces. In: Clinical immunology, ed. Rich R.T, Mosby Elsevier, Philadelphia, 2008, pp. 287-304.

6. Green W. B., K. Eaton, S. Krakowka. Porcine gastric mucosa associated lymphoid tissue (MALT): stimulation by colonization with the gastric bacterial pathogen, Helicobacter pylori. – *Vet. Immunol. Immunopathol.*, **56**, 1997, 119-131.

7. Kiyono, H., J. Kunisawa, J. R. McGhee, J. Mestecky. The mucosal immune system. In: Fundamental immunology, editor Paul W.E., Lippincott Williams & Wilkins, Philadelphia, 2008, pp. 983–1030

8. Liebler-Tenorio, E. M. R. Pabst. MALT structure and function in farm animals. – *Vet. Res.*, **37**(3), 2006, 257-280.

9. McGhee, J. R., K. Fujihashi. Inside the Mucosal Immune System. *PLOS Biology,* **10**(9), 2012, e1001397. doi:10.1371/journal.pbio.1001397

10. Morfitt, D. C., J. F. L. Pohlenz. Porcine colonic lymphoglandular complex: distribution, structure, and epithelium. – *Am. J. Anat.*, **184**, 1989, 41-51.

11. Neutra, M. R., T. L. Phillips, E. L. Mayer, D. J. Fishkind. Transport of membrane-bound macromolecules by M cells in follicle-associated epithelium of rabbit Peyer's patch. – *Cell Tissue Res.*, **247**(3), 1987, 537-546.

12. Nomina Histologica Veterinaria. 1st eddition. International Committee on 5 Veterinary Histlogical Nomenclature (I.C.V.H.N.) to the World 6 Association of Veterinary Anatomists. Published on the website of the World 7 Association of Veterinary Anatomists, 2017, pp. 30, 31, 51.

13. Torres-Medina, A. Morphologic characteristics of the epithelial surface of aggregated lymphoid follicles (Peyer's patches) in the small intestine of newborn gnotobiotic calves and pigs. – *Am. J. Vet. Res.,***42**, 1981, 232-236.

14. Stefanov, I. S. Age-dependent distribution and size of lymphatic nodules as components of extrahepatic bile ductassociated lymphatic tissue in domestic swine – a micromorphometric study. *Bulg. J. Vet. Med*., ONLINE FIRST ISSN 1311-1477, 2021, DOI: 10.15547/bjvm.2021-0003

15. Urmila, T. S, P. J. Ramayya, M.S. Lakshmi, A. V. N. Siva Kumar. Histomorphological studies on Gut Associated Lymphoid Tissue of pig (Susscrofa). – *Pharma Innov. J.*, **8**(3), 2019, 97-101.

Table1. Diameter, height (h) and width (w), in μm, of solitary lymphatic nodules (SLNs) and aggregated lymphatic nodules (ALNs) in the wall of gallbladder (*Vesica fellea*).

Parameters	3 year-old pigs Mean±SD	6 month-old pigs Mean±SD	2 month-old pigs Mean±SD
Diameter of SLNs			
Vesica fellea:			
• fundus	382.0±15.23	360.7 ±8.88**D1**	-
• corpus	527.1± 27.18**C4**	370.9± 32.70**D4**	-
• collum	638.6 ±20.88**A4,B4**	396.7 ±13.90**B3,D4**	-
Height and width of SLNs			
• fundus (h)	414.5± 17.40	394.8 ±23.42	-
(w)	349.5±19.34	326.6±18.26	-
• corpus (h)	591.5 ±45.29	373.3 ±47.02	-
(w)	462.7±17.54	368.5±35.31	-
• collum (h)	663.7 ±39.74	418.7 ±21.08	-
(w)	613.50±0.32	374.7±22.83	-
Diameter of ALNs			
Vesica fellea:			
• fundus	628.6± 6.5	-	-
• corpus	805.8 ± 19.78 **C4**	930.2± 7.43 **D4**	-
• collum	820.5±24.63**A4,B4**	1008.0±10.47**A4,D4**	-

A4 ($P < 0.0001$)statistical significant difference between *collum* and *corpus vesicae felleae*
B 3,4 ($P < 0.001$, $P < 0.0001$)statistical significant difference between *collum* and *fundus vesicae felleae*
C4 ($P < 0.0001$) statistical significant difference between *corpus* and *fundus vesicae felleae*
D 1,4 ($P < 0.05$, $P < 0.0001$) statistical significant difference between 6 month- and 3 year- old animals

Table 2. Number (per cross section) of solitary lymphatic nodules (SLNs) in the wall of *Vesica fellea*

Parameters	3 year-old pigs Mean±SD	6 month-old pigs Mean±SD	2 month-old pigs Mean±SD
Number of SLNs			
Gallbladder:			
✓ fundus	8.50± 0.51	11.11±0.83 **D4**	-
✓ corpus	11.06± 0.80 **C4**	13.89±0.83 **C4,D4**	-
✓ collum	6.11± 0.83 **A4, B3**	7.00±0.77 **A4,B4,D2**	-

A 4 ($P < 0.0001$) statistical significant difference between *collum* and *corpus vesicae felleae*
B 3,4 ($P < 0.001$; $P < 0.0001$) statistical significant difference between *collum* and *fundus vesicae felleae*
C 4$P < 0.0001$) statistical significant difference between *corpus* and *fundus vesicae felleae*
D 2,4 ($P < 0.01$; $P < 0.0001$) statistical significant difference between 6 month- and 3-year-old animals

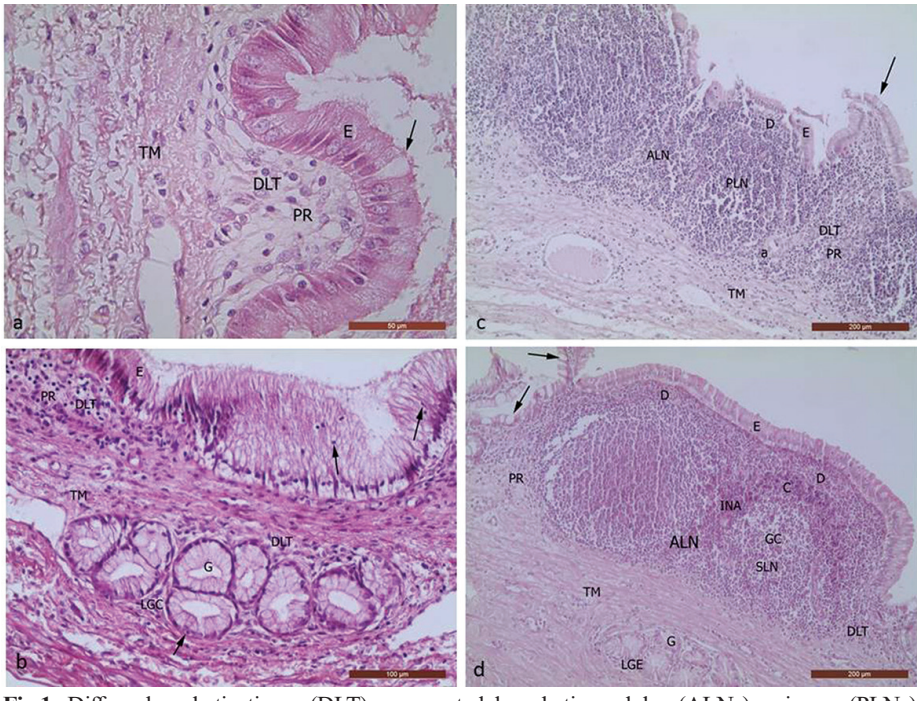

Fig.1. Diffuse lymphatic tissue (DLT), aggregated lymphatic nodules (ALNs), primary (PLNs) and secondary lymphatic nodules (SLNs) as components of gallbladder-associated lymphoid tissue.

a,b – *fundus* and *collum,* respectively of gallbladder in 2-month-old pigs

c – *corpus* of gallbladder in 6-month-old pigs

d – *collum* of gallbladder in 3-year-old-animals

C – *corona* lymphonoduli; GC – germinal center of the nodule, a –arteriola; INA – internodular area; D – dome; LGE – lymphoglandular epithelium; E – *lamina epithelialis mucosae*; PR – *lamina propria mucosae*; TM – *tunica muscularis*; G – *glandulae vesicae felleae*; arrows (a,c,d) – goblet cells (b) – intraepithelial lymphocytes.

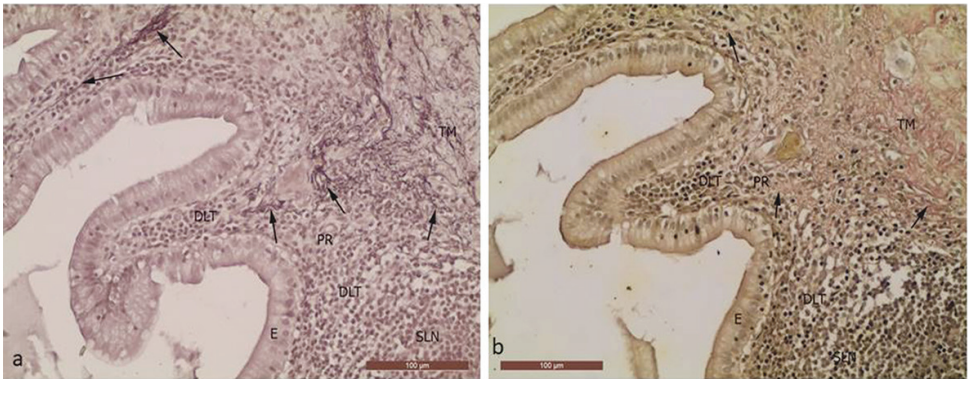

Fig. 2. Elastic (a, arrows) and collagen fibers (b, arrows) within the lymphatic tissue of gallbladder in 6 month-old pigs.

DLT – diffuse lymphatic tissue, SLN – solitary lymphatic nodule,

E – *laminae pithelialis mucosae*; PR – *lamina propria mucosae*; TM – *tunica nuscularis*;