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Original article

AGE-DEPENDENT DISTRIBUTION AND SIZE OF LYMPHATIC NODULES AS COMPONENTS OF EXTRAHEPATIC BILE DUCT-ASSOCIATED LYMPHATIC TISSUE IN DOMESTIC SWINE – A MICROMORPHOMETRIC STUDY

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Summary

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Due to the biological and immunological similarity between pigs and humans, pigs are used in medical research. Many morphological studies regarding the structure of the components of mucosal associated lymphatic tissue have been performed in animals including pigs as well as in humans. However, no any information about the existence of extrahepatic bile duct-associated lymphoid tissue (EHBDALT) is available in pigs. The aim of this work was to study the distribution and size of lymphatic nodules in the wall of extrahepatic bile ducts of immature and mature pigs in order to describe the structure of EHBDALT as component of mucosa-associated lymphoid tissue. It was found out that EHBDALT consisted of diffuse lymphatic tissue, solitary and aggregated lymphatic nodules. In 2-month-old pigs, diffuse lymphatic tissue predominated but single lymphatic nodules were found as inactive homogenous encapsulated aggregates of lymphocytes. In mature animals, diffuse lymphatic tissue, primary and secondary nodules were observed. The present micromorphometric study allowed evaluating the age-dependent distribution and size of lymphatic nodules in porcine extrahepatic bile ducts. It provides original data on the presence and age-dependent structure of bile duct MALT in domestic pigs.

Key words: extrahepatic bile ducts, lymphatic tissue, morphometry, pigs

INTRODUCTION

Mucosa-associated lymphoid tissue (MALT) contains T cell regions, B cell areas with a high frequency of surface IgA-positive (sIgA+) B cells, and a subepithelial area with antigen-presenting cells (APCs), including dendritic cells (DCs) for the initiation of specific immune responses. The MALT is covered by a subset of differentiated microfold (M) cells, but not goblet cells, and underlying lymphoid cells that play central roles in the initiation of mucosal immune responses. M cells take up antigens (Ags) from the lumen of the intestinal and nasal

mucosa and transport them to the underlying DCs. The DCs carry Ags into the inductive sites of the Peyer's patch or through draining lymphatic vessels into the mesenteric lymph nodes (MLNs) for initiation of mucosal T and B cell responses (Campbell & Butcher, 2002; Iwata *et al.*, 2004). Liebler-Tenorio & Pabst (2006) described the structural characteristics of MALT. Lymphoid tissue is localised near the mucosal surface. It consists of organised lymphoid tissue with lymphoid nodules (follicles, LNs) and interfollicular areas, which is T cell-dependent. The LNs (primary and with germinal centres) consist of B-lymphocytes, follicular dendritic cells (FDC), CD4+ T-lymphocytes and macrophages. MALT is represented by both isolated solitary lymphoid nodules (SLN) and larger aggregates (ALNs) consisting of several LNs. The epithelium overlying MALT is infiltrated by lymphocytes forming lymphoepithelium (LE). MALT contains lymphatic vessels which transport immune cells and antigens to regional lymph nodes amplifying the immune responses (Brandtzaeg & Pabst, 2004).

Pabs (2020) reported that the pig is an omnivorous, monogastric species with many advantages to use it as an animal model for human diseases. The porcine immune system is similar to human one for more than 80% of analysed parameters in contrast to that of mice with only about 10% (Liebler-Tenorio & Pabst, 2006). According to the anatomical localisation, MALT structures are subdivided into: conjunctiva-associated lymphoid tissue (CALT), lacrimal drainage-associated lymphoid tissue (LADLT), salivary gland-associated lymphoid tissue/duct-associated lymphoid tissue (SALT/DALT), nose or nasopharynx-associated lymphoid tissue (NALT), lymphoid tissues of Waldeyer's ring, larynx-associated lymphoid tissue (LTALT), bronchus-associated lymphoid tissue (BALT), gastric mucosa-associated lymphoid tissue (gastric MALT), gut-associated lymphoid tissue (GALT) (Brandtzaeg & Pabst, 2004).

In pigs, some parts of MALT such as Waldeyer's ring, BALT and GALT have been often subject of reserach and therefore, well-studied. However, in animals including domestic swine, there is no information in the literature (including Nomina Histologica Veterinaria, 2017) about the presence and components of the lymphoid tissue in extrahepatic bile ducts (common hepatic duct – *ductus hepaticus communis,* DHC; cystic duct – *ductus cysticus,* DC and common bile duct – *ductus choledochus,* DCH).

The present study was undertaken to study the distribution and size of LNs in the wall of extrahepatic bile ducts of pigs at the age of 2 months, 6 months and 3 years in order to describe the histological structure of extrahepatic bile duct associated lymphatic tissue (EHBDALT) as component of MALT in domestic pigs.

MATERIALS AND METHODS

Material

The present study was conducted on extrahepatic bile ducts (common hepatic duct, common bile duct and cystic duct) and major duodenal papilla of 18 pigs grouped as follows: 6 pigs at 2 months of age, 6 pigs at 6 months and 6 pigs at 3 years, with weight 22–33 kg for the pigs of the first group, 92–100 kg (second group) and 280–300 kg (third group). The animals were used under Scientific Project number 13/2017, Medical Faculty, Trakia University, Stara Zagora, Bulgaria. The tissue samples were collected from pigs

slaughtered for meat consumption. Cystic duct was subdivided into initial, middle and terminal parts. First, the common bile duct was separated into two main parts: extra- and intramural (intraduodenal) parts, then the extramural part was additionally subdivided into initial, middle and terminal segments. The tissue samples were fixed in 10% aqueous solution of formalin. Later, these samples were dehydrated in alcohols and embedded in paraffin. About 5–6 μm thick paraffin serial cross sections were obtained. Tissue sections were mounted on gelatinised slides, twice placed in xylene and rehydrated by decreasing ethanol concentrations. Then the sections were stained with haematoxylin and eosin.

Histochemical study for identification of collagen and elastic fibres

Another part of the sections were stained using Van Gieson's method in order to visualise collagen fibres (in red) and with Orcein dye – for detection of elastic fibres (in dark brown).

Morphometric study

The measurements were performed by light microscope (LEICA DM1000) with a digital camera (LEICA DFC 290) and software (LAS V4.10.0 2016) on serial cross sections in order to define the size (diameter, height and width of nodules corresponding to their long- and short axis diameter in μm) and density (number per cross section) of lymphatic nodules.

Statistical analysis

The data were processed by GraphPad Prism 6 for Windows (GraphPad Software, Inc., USA) via one-way analysis of variance (one-way ANOVA) followed by Tukey-Kramer's post-hoc test and are presented as mean ± standard deviation. P values smaller than 0.05 were considered statistically significant.

The terminology was consistent with Nomina Histologica Veterinaria (2017).

RESULTS

The histological study showed that extrahepatic bile duct-associated lymphoid tissue was represented by diffuse lymphatic tissue (DLT), solitary primary or secondary lymphatic nodules (*nodule lymphatici solitarii*) and aggregated lymphatic nodules (*nodule lymphatici aggregati*) (ALNs) localised in the wall of extrahepatic bile ducts represented by DHC, DC and DCH terminating with a major duodenal papilla (Fig. 1–3).

In 2-month-old pigs, LNs in DHC, DC and DCH were of primary type only. In 6-month-old pigs, LNs were of primary and secondary type, but in 3-year-old animals, the secondary LNs predominated.

The lymphatic tissue in DC of 2-month-old pigs was represented by DLT only in its initial and middle part and by both DLT and solitary primary LNs in its terminal part (Fig. 1A, B, C). The DLT in the initial and middle part of DC was localised in the propria (*lamina propria mucosae*) and *tunica muscularis* (TM) around glands and was looser than that in the terminal part of DC. The terminal part of the duct showed also the presence of solitary LNs (horizontally oriented), localised deep in the TM near the subserous layer (*tela subserosa*). Primary LNs predominated because the germinal centre (or medulla) and dense corona (or cortex) were not clearly defined. The LNs were encircled by delicate connective tissue capsules. Their diameter in 2-month-old pigs was the biggest, followed by 6-month- and 3-year-old animals (Table 1).

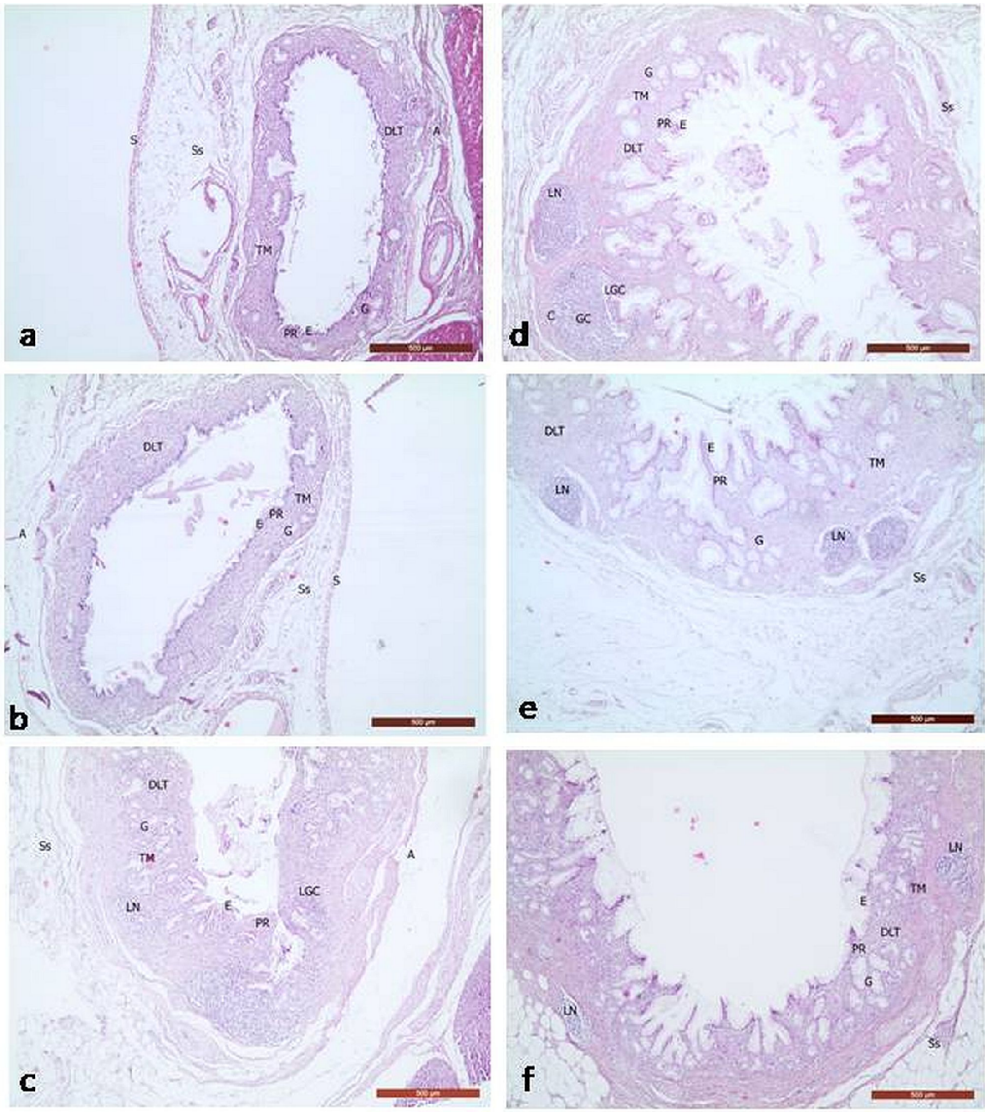

Fig. 1. Representative cross sections of initial, middle and terminal part of cystic duct in 2-month-old (A, B, C, respectively) and in 6-month- old pigs (D, E, F, respectively). **A, B, C**: In the propria (PR) and *tunica muscularis* (TM) of initial and middle part, diffuse lymphatic tissue (DLT) only is present. Lymph nodules (LN) together with DLT are observed in the terminal part of cystic duct. Some glandular units (G) and lymphatic tissue form lymphoglandular complexes (LGC). **D, E, F:** LNs together with DLT are present in the PR and TM of cystic duct at all ages. E – *lamina epithelialis mucosae*, S – *tunica serosa*, Ss – *tela subserosa*. H & E staining, bar=500 µm.

Table 1. Diameter (d), height (h) and width (w) (mean ± SD, in μm) of solitary lymphatic nodules in the wall of *Ductus cysticus*, *Ductus hepaticus communis* (DHC), *Ductus choledochus* (DCH) and *Papilla duodeni major* (PDM)

Parameters		3 year-old pigs	6 month-old pigs	2 month-old pigs
Ductus cysticus				
initial part	d	349.3± 16.24	425.7± 34.05^{D4}	–
	h	289.0 ± 8.43	328.3 ± 37.48	–
	w	409.6±32.08	523.2 ±44.52	306.4± 50.09^{D4}
middle part	d	228.6± 7.22^{C4}	221.6± 23.21$^{C4\ ,D0}$	–
	h	211.1 ± 7.65	220.7 ± 38.43	–
	w	246.0 ±14.02	226.9 ±16.79	–
terminal part	d	179.2± 9.67^{A4,B4}	226.2±26.79^{A0,B4,D4}	306.4± 50.09^{D4}
	h	289.0 ± 8.43	196.6 ± 11.33	238.5 ± 52.96
	w	196.4 ±16.81	254.9 ±51.83	374.3 ±67.29
Ductus hepaticus communis				
	d	429.3±16.14	386.8± 16.17^{E4}	–
	h	489.1±15.41	299.3 ± 13.63	–
	w	369.5± 27.04	474.3 ±31.96	–
Ductus choledochus – extramural part				
initial segment	d	351.6± 21.30	314.7± 9.61K3	–
	h	361.6±29.06	330.6 ±16.54	–
	w	341.5± 33.78	298.7± 6.96	–
middle segment	d	202.3± 6.0^{N4}	289.7± 10.79^{N0,O4}	395.8±4.42^{N4,O4,P4}
	h	230.1 ±7.80	317.0 ±16.20	289.0 ± 7.73
	w	174.4± 14.67	262.4 10.33	502.7 ±7.45
terminal segment	d	–	434.9 ±23.16^{M4,O4}	300.2±23.88^{M4,O4,P4}
	h	–	413.6 ± 45.87	416.2 ±45.65
	w	–	461.4 ±15.78	184.1± 6.30
Ductus choledochus – intramural part				
	d	192.2±30.38^{J4,K0,L4}	326.9±19.76^{J4,K4,L0,O4}	331.0±22.91^{J2,K4,L4,O4,P0}
	h	248.5± 29.91	272.7 ± 18.24	298.1 ± 20.94
	w	126.4± 7.48	381.0 ±30.93	363.8 ±40.41
Papilla duodeni major				
	d	279.2±24.14	627.9 ±55.28^{Q4}	228.1± 18.65$^{Q4\ ,R4}$
	h	188.2 ± 22.44	537.7 ± 39.34	181.1 ± 39.13
	w	370.3±52.00	718.1 ±103.3	275.1±17.81

Letters indicate statistical significant differences between: (A) terminal and middle part of cystic duct; (B) terminal and initial part of cystic duct; (S) middle and initial part of cystic duct; (D) 6-month and 3-year old animals; between 2-month old pigs and other two age groups; (E) common hepatic duct of 6- month and 3-year-old animals; (F) intramural part and terminal segment of DCH; (G) intramural part and middle segment of DCH; (H) intramural part and initial segment of DCH; (I) terminal segment and middle segment of DCH; (J) middle segment and initial segment of DCH; (K) common bile duct of 6-month and 3-year-old animals; between 2-month and 3-year-old animals; (L) common bile duct of 2-month and 6-month-old pigs; (M) PDM of 6-month and 3-year-old animals; between 2-month pigs and 3-year-old animals; (N) PDM of 2-month and 6-month-old pigs. Numbers indicate level of statistical significance: (0) $P>0.05$; (1) $P<0.01$; (2) $P<0.001$; (3) $P<0.0001$.

Table 2. Density (mean number per cross section ± SD) of solitary lymphatic nodules in the wall of *Ductus cysticus*, *Ductus hepaticus communis* (DHC), *Ductus choledochus* (DCH) and *Papilla duodeni major* (PDM)

Parameters	3-year-old pigs	6-month-old pigs	2-month-old pigs
Ductus cysticus			
initial part	2.50±0.51	5.39±1.29^{D4}	–
middle part	1.28±0.46^{C4}	2.72±0.46^{C4,D4}	–
terminal part	1.22±0.43$^{A0\ ,B4}$	6.00±0.69$^{A4,\ B0,\ D4}$	2.17±0.79$^{D4,\ E4}$
Ductus hepaticus communis			
	3.50 ±0.51	4.06 ±0.64^{F2}	–
Ductus choledochus – extramural part			
initial segment	2.56±0.51	3.56±0.51^{P4}	–
middle segment	4.56±0.51^{K4}	4.67±0.49^{K4,P0}	1.50±0.51^{K4,L4,M4}
terminal segment	–	12.44±0.70$^{J4\ ,L4}$	3.94±0.64^{J4,L4,M4}
Ductus choledochus – intramural part			
	2.61±0.50^{G4,H4,I0}	8.22±0.81^{G4,H4,I4,P4}	8.00±0.77^{G4,H4,I4,P4,M0}
Papilla duodeni major			
	4.33 ±0.77	4.67± 0.49^{N0}	5.22± 0.73$^{R3,\ O0}$

Letters indicate statistical significant differences between: (A) terminal and middle part of cystic duct; (B) terminal and initial part of cystic duct; (C) middle and initial part of cystic duct; (D) 6-month and 3-year-old animals; between 2-month-old pigs and other two age groups; (E) common hepatic duct of 6-month- and 3-year-old animals; (F) intramural part and terminal segment of DCH; (G) intramural part and middle segment of DCH; (H) intramural part and initial segment of DCH; (I) terminal segment and middle segment of DCH; (J) middle segment and initial segment of DCH; (K) common bile duct of 6-month and 3-year-old animals; between 2-month and 3-year-old animals; (L) common bile duct of 2-month and 6-month-old pigs; (M) DCH of 6-month- and 2-month-old animals; between 2-month pigs and 3-year-old animals; (N) PDM of 2-month and 3-year-old; between 6-month and 3-year-old animals (O) PDM of 2-month- and 6-month-old pigs. Numbers indicate level of statistical significance: (0) $P>0.05$; (1) $P<0.01$; (2) $P<0.001$; (3) $P<0.0001$.

The number of LNs in the terminal part of the DC was higher than in 3-year-old animals but lower than that in 6-month-old pig (Table 2).

LNs were observed near the glands of DC forming lymphoglandular complex (LGC) (Fig.1C, D). LGCs were localised in mucosal and muscular layers composed of LNs and internodular lymphatic tissue penetrated by extensions of cystic duct's glands. LGC epithelium (also known as nodule associated epithelium) consisted of secretory glandular cells, intraepithelial leukocytes and single goblet cells. The capsule of LNs forming LGC was interrupted by adjacent glands in all studied bile ducts (Fig. 2A, B, C and Fig. 3D, F). The LNs were oval shaped with a height smaller than the width. They were the largest in the initial part, following by the middle and terminal parts of DC in 6-month- and 3-year-old animals. The number of LNs was the highest in the terminal part, followed by initial part and the lowest in the middle part of DC. The DLT was localised predominantly in the propria but it was also present in TM near and between glands.

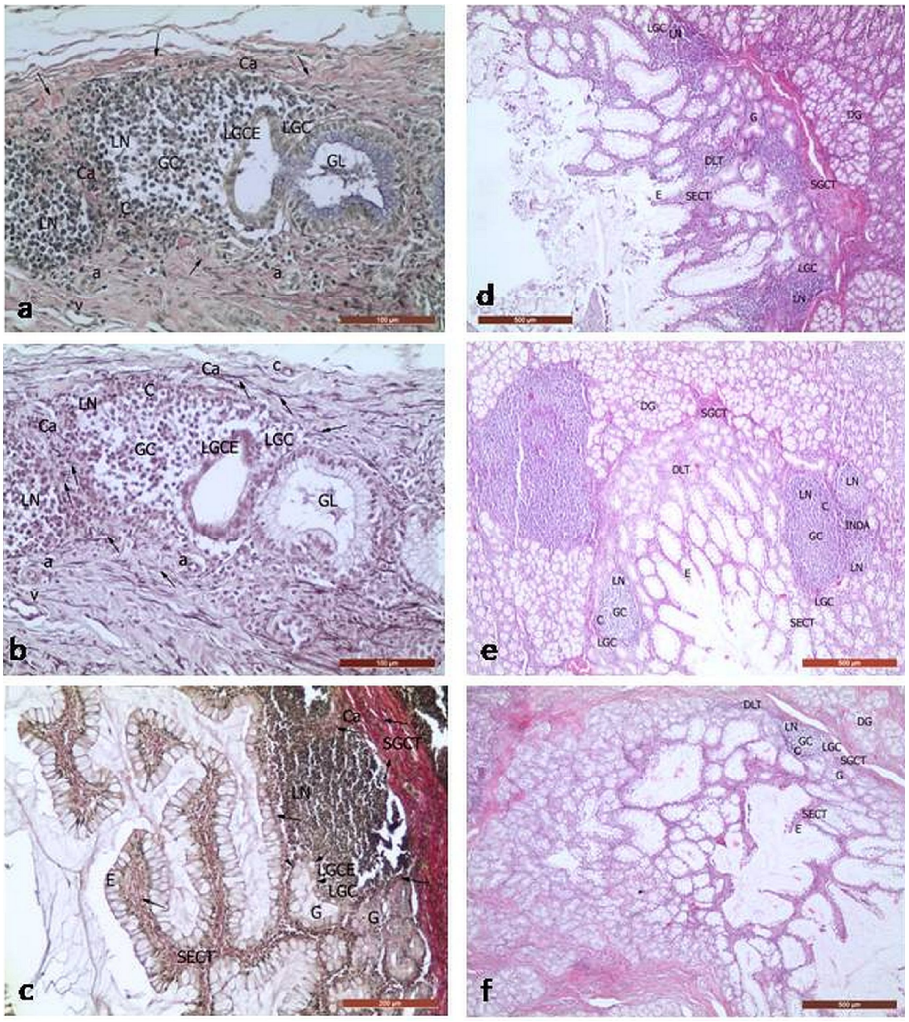

Fig. 2. Representative cross sections of the middle part of common bile duct in 6-month-old pigs (A, B, C) and of the major duodenal papilla in 2-month-old (d), 6-month-old (e) and 3- year-old pigs (f). **A,B:** Cross section trough middle part of common bile duct of 6-month-old pigs, Van Gieson (a) and Orcein staining (b), bar=100 µm. Lymphatic nodules (LN) are localised in *Tunica muscularis* and encircled by connective tissue capsule (Ca) containing collagen (A - arrows), elastic fibres (B - arrows) and vessels – capillaries (c), arterioles (A,B - a) and venules (v). The capsule of some LN is interrupted by adjacent glands (GL) forming lymphoglanular complex (LGC). LGC has specific lymphoglandular complex epithelium (LGCE) represented mainly by secretory glandular cells and intraepithelial leukocytes; **C:** Cross section trough major duodenal papilla of 6- month-old pigs, Van Gieson staining, bar=200 µm. LGC contains LGCE represented by secretory glandular cells, intraepithelial leukocytes and single goblet cells (arrowheads). Ca – capsule of LN containing collagen fibres (arrows). Arrows – collagen fibres in SECT, SGCT and in LN capsule; E – *lamina epithelialis mucosae*; **D,E,F:** cross section trough major duodenal papilla of 2-month-, 6-month- and 3-year-old pigs, respectively, H&E staining, bar=500 µm. C – corona of secondary LN, GC – germinal centre of secondary LN, DLT - diffuse lymphatic tissue; E – *Lamina epithelialis mucosae*, SECT – subepithelial connective tissue layer, SGCT – subglandular connective tissue layer, INDA – internodular lymphatic area, G - biliary glands of major duodenal papilla, DG – duodenal glands.

ALNs were identified only in the initial and terminal part of DC in pigs at the age of 6 months but no LAs observed in 2-month- and 3-year-old animals. ALNs were localised in TM. In 6-month-old animals, the diameter of ALNs in the initial part was bigger than that in the terminal part of the DC (P<0.0001) (Table 3).

In 3-year-old animals, the DLT predominated compared to LNs. The LNs were observed near the glands forming LGC. In 3-year-old pigs, the diameter and

Table 3. Diameter (d), height (h) and width (w) (mean ± SD, in μm) of aggregated lymphatic nodules in the wall of *Ductus cysticus*, *Ductus hepaticus communis* (DHC), *Ductus choledochus* (DCH) and *Papilla duodeni major* (PDM)

Parameters		3-year-old pigs	6-month-old pigs	2-month-old pigs
Ductus cysticus:				
initial part	d	–	643.7± 11.52	–
	h	–	372.6 ±19.69	–
	w	–	643.7±11.52	–
middle part	d	–	–	–
	h	–	–	–
	w	–	–	–
terminal part	d	–	558.0±14.26[E4]	–
	h	–	327.3±10.87	–
	w	–	788.6±25.80	–
Ductus hepaticus communis				
	d	–	–	–
	h	–	–	–
	w	–	–	–
Ductus choledochus – extramural part				
initial segment	d	–	–	–
	h	–	–	–
	w	–	–	–
middle segment	d	–	–	–
	h	–	–	–
	w	–	–	–
terminal segment	d	–	1198.00±36.91	–
	h	–	622.6±9.21	–
	w	–	1773.0± 73.56	–
Ductus choledochus – intramural part				
	d	–	–	591.9±3.06
	h	–	–	402.9±4.93
	w	–	–	780.8±5.76
Papilla duodeni major				
	d	–	1071.0±46.94[F4, G4]	740.6±13.15[H4]
	h	–	648.1±9.43	414.5±7.18
	w	–	1494.0±89.31	1067.0±27.30

(4) statistical significant difference at P<0.0001 between: (E) initial and terminal part of cystic duct; (F) PDM of 2-month and 6-month-old pigs; (G) PDM and terminal segment of extramural part of common bile duct; (H) PDM and intramural part of common bile duct.

number of LNs were the biggest in the initial part, followed by the middle and terminal parts of DC. In all DC parts in this age group, the number of LNs was lower than in 6-month-old pigs. The shape of LNs was oval with a height smaller than the width. In 3-year-old animals, the diameter of LNs was slightly lower than in 6-month-olds.

The lymphatic tissue in DHC of 2-month-old pigs was represented mainly by well-developed DLT filling the propria and TM. The DLT tissue was surrounded the glands and was localised between the glands and formed LGCs localised in mucosal and muscular layers. LGCs were composed of DLT penetrated by extensions of biliary glands. LGC epithelium consisted of goblet cells, secretory glandular cells, individual and clustered intraepithelial leukocytes. Lymphatic nodules were not observed.

In 6-month-old pigs, the lymphatic tissue of DHC was presented by both DLT and solitary LNs. The DLT filled the propria and TM and were localised around the glands. LNs were found deep in TM near the subserous layer. Single encapsulated LNs also were detected in the subserosal layer. They were oval (horisonal) in shape with height smaller than width. Their diameter in 6-month-old pigs was smaller than in 3-year-old animals, but their number in 6-month-old pigs was higher than in 3-year-old animals.

LGCs consisted of well-defined masses in muscle layer composed of lymphatic nodules and internodular DLT penetrated by branching extensions of biliary glands. LGC epithelium consisted of secretory glandular cells, single goblet cells, individual and clustered intraepithelial leukocytes.

The initial part of DCH of 2-month-old pigs showed well developed DLT around and between glands in propria and TM (Fig. 2 and 3). Solitary LNs were not present. In the middle and terminal segment of extramural DCH part, DLT in propria and TM was observed. Solitary primary LNs were localized in single row deep in TM near the subserous layer. The shape of LNs was oval with height smaller than the width in the middle segment and intramural part but with width smaller than the height in the terminal segment of the duct. Their diameter was the highest in the middle segment, followed by terminal segment of extramural DCH part and intramural DCH part. The number of LNs was the highest in the intramural part of DCH, followed by terminal and middle segment of the extramural part of DCH. ALNs were present in the intramural part of the duct only. ALNs were localised in TM. In 2-month old animals, the diameter of ALNs in the DCH was smaller than that in PDM (Fig. 3).

In 6-month-old pigs, the initial segment of the extramural part of DCH contained diffuse lymphatic tissue in propria and TM without LNs. In the middle segment of extramural part of common bile duct, the DLT showed the same localisation, but LNs are presented in TM arranged in one row. In the terminal segment LNs were in the highest number and arranged in three rows. In TM, ALNs forming structures similar to intestinal patches were defined (Fig. 3). A nodule contained a clearly visible germinal centre and dense corona. The LNs were encircled by delicate connective tissue capsule consisting of collagen and elastic fibres and vessels belonging to microcirculatory bed (Fig. 2 and 3). The DLT was observed in the spaces between nodules. In the intramural part of DCH, LNs were arranged in two layers (Fig. 3). The LNs were observed in the subepithelial layer of

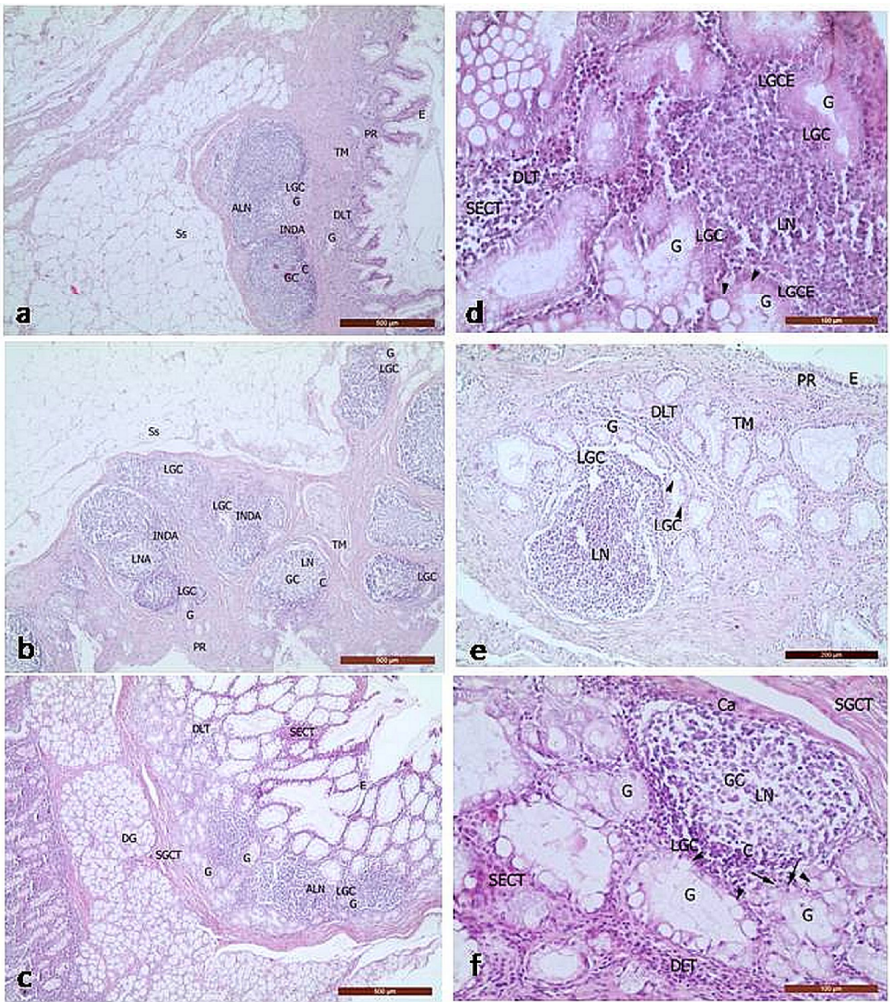

Fig. 3. Representative cross sections trough the initial part of cystic duct (A), terminal part of extrahepatic common bile duct (B), and intramural part of common bile duct (C) in 6-month-old pigs and cross sections trough major duodenal papilla of 2-month-old (D), initial part of common bile duct of 6 month-old (E) and major duodenal papilla of 3-year-old pigs (F). **A,B,C:** aggregated lymphatic nodules (ALN) in the initial part of cystic duct, terminal part of extrahepatic common bile duct, and intramural part of common bile duct Ss – *tela subserosa*, LN – lymphatic nodule, INDA – internodular lymphatic area, DG – duodenal glands. H &E staining, bar=500 µm. **D,E,F:** lymphatic tissue in major duodenal papilla of 2-month-old pigs, initial part of common bile duct of 6-month-old pigs and major duodenal papilla of 3-year-old animals, respectively. Lymphoglandular complex (LGC) has specific lymphoglandular complex epithelium (LGCE) represented by secretory glandular cells, intraepithelial leucocytes (f – arrows) and single goblet cells (arrowheads), DLT – diffuse lymphatic tissue, SECT – subepithelial connective tissue layer, SGCT – subglandular connective tissue layer, G – biliary glands, Ca – capsule of LN, C – corona of secondary LN, GC – germinal center of secondary LN, E – *lamina epithelialis mucosae*, PR – *lamina propria mucosae*, TM – *tunica muscularis*. H&E staining, bar=100 µm (D), bar=200 µm (E), bar = 100 µm (F).

propria and in subglandular layer of propria near the glands forming LGCs. LGCs were located in mucosal and muscle layers of DCH containing LNs and internodular lymphoid tissue penetrated by branching extensions of biliary glands. LGC epithelium consisted of single goblet cells, secretory glandular cells and intraepithelial leukocytes (Fig. 2 and 3). LNs showed pale germinal centre and dense corona. They were oval with height bigger than width in the initial and middle segment of the extramural part of DCH but with width exceeding the height in the terminal extramural segment and in intramural DCH part. The diameter of LNs was the highest in the terminal segment of extramural part of DCH, followed by intramural part of DCH, initial and middle part of extramural DCH. The number of LNs increased from initial segment to the terminal segment of extramural DCH part. In the intramural part of DCH, their number was lower than terminal segment, but bigger than middle and initial segment of extramural part of DCH.

ALNs were localised in the DCH terminal segment only and were represented by 3–7 LNs. They were located in the TM. In 6-month-old animals, the diameter of ALNs in the DCH was bigger than in PDM.

In 3-year-old animals, the DLT predominated compared to LNs in all parts of DCH. The single LNs were observed in the cranial and middle segment of extramural part of DCH, but there were no nodules in the terminal extramural DCH segment and in the intramural part of DCH. The germinal centre and dense corona of LNs were defined. The shape of LNs was oval with height smaller than width. The diameter of LNs decreased to the terminal segment of the extramural DCH part. It was the largest in the initial segment, followed by the middle segment of the extramural part of DCH and the smallest – in the intramural part of DCH. The number of LN in the middle segment of DCH was the highest.

In the intramural part of DCH, the LNs were arranged in two layers. Some LNs showed pale germinal centre and dense corona. LNs were oval with height bigger than the width. Their diameter and number were the lowest comparing to 6-month- and 2-month-old pigs. The lymphatic nodules were observed near the glands forming LGC. LGCs consisted of well-defined mucosal masses composed of LNs and internodular DLT penetrated by branching extensions of biliary glands. LGC epithelium consisted of goblet cells, secretory glandular cells, individual and clustered intraepithelial leukocytes.

Therefore in extrahepatic bile ducts an age-dependent regression in LNs number and size was detected. In 2-month-old pigs, primary LNs were found as small encapsulated aggregates of lymphocytes. In 6-month-old pigs both primary and secondary LNs were present but their number and size decreased with age. ALNs in all age groups were localised in TM and represented by 2–7 nodules depending on the age.

Morphologically, ALNs, very similar to PPs, in the terminal segment of DCH were separated into three main domains: the nodular area, the internodular area and the nodule-associated epithelium. The internodular area was found to contain internodular DLT, connective tissue fibres (collagen and elastic fibres) and vessels of microcirculatory bed (capillaries, arterioles and venules) (Fig. 2A, B).

LGCs were observed in TM mainly and consisted of well-defined lymphatic nodules and internodular lymphoid tissue penetrated by branching extensions of

biliary glands. LGC epithelium also known as nodule-associated epithelium contained single goblet cells, glandular secretory cells and intraepithelial leukocytes (Fig. 2 and 3).

In PDM of 2-month-old pigs, the DLT predominated compared to primary LNs arranged in one row. In some pigs single LNs were observed in subglandular connective tissue layer. The shape of LNs was oval with a height smaller than the width. In this group, their diameter was the smallest but their number was the highest, followed by 6-month- and 3-year-old animals.

ALNs were present in 2-month- and 6-month-old pigs but not in 3-year-old animals. ALNs were represented by 2–3 LNs and localised in the propria between deep and superficial glands reaching the base of mucosal folds. In 2-month-old animals, ALNs were smaller than in intramural part of DCH.

In 6-month-old pigs, secondary LNs were arranged in two rows forming aggregates near the glands. LNs showed pale germinal centre and dense corona. The DLT was also well developed and localised between LNs and around glands. ALNs were localised between glands of propria and in subglandular connective tissue. They formed lymphoepithelium and LGCs. The shape of LN was oval with a height smaller than the width.

In 6-month-old pigs the diameter of ALNs was bigger than in 2-month-old pigs ($P<0.0001$).

Similar to DCH, the DLT in PDM predominated compared to secondary LNs in 3-year-old animals, with pale germinal centre and dense corona. Small solitary LNs were present in subglandular connective tissue layer of prorpria. The shape of LNs was oval with a height smaller than the width. ALNs were not observed.

DISCUSSION

Based on the anatomical localisation, Brandtzaeg & Pabst (2004) reported that MALT structures can be subdivided into CALT, LADLT, SALT/DALT, NALT, LTALT, BALT, gastric MALT, GALT. However, in animals including domestic swine, there is no scientific information regarding the presence of well-developed lymphatic tissue and its structural components in extrahepatic bile ducts. Therefore, the present histomorphometric study provided for the first time information about the existence of EHBDALT as another component of MALT and discussed its structural characteristics. EHBDALT was represented by DLT, solitary (primary and secondary) LNs and ALNs. The results of our study regarding histological structures of EHBDALT in 2-month-, 6-month- and 3-year-old pigs were compared to GALT in pigs and other animal species. In extrahepatic bile ducts, age-depending changes in number and size of LNs was detected. In general, the number and size of LNs were the highest in 6-month-old animals and decreased with age. ALNs were not observed in DHC from all age groups. Also ALNs were not present in bile ducts of animals at the 3 years of age.

The results of the present study showed that the lymphoid tissue in the extrahepatic bile ducts of 2-month-old pigs was formed by DLT and primary LNs. However, in the intramural part of DCH and in PDM the lymphoid tissue included also lymphatic aggregates. In 2-month-old pigs, primary LNs were found as small encapsulated aggregates of lymphocytes. In 6-month-old pigs secondary LNs were present but their number and size decreased with age. ALNs in all age groups were localised in TM and repre-

sented by 2–7 LNs depending on the age. The current study showed that the lymphatic tissue developed until six months of age. No signs for lymphatic tissue involution were seen in the EHBDALT at the age of six months. However, such involution was detected at the age of 3 years.

In the current study, in 2-month-old animals, the primary LNs in bile ducts were without germinal centre, but in mature pigs the secondary nodules showed germinal centre. The secondary LNs in bile duct showed pale germinal centre and dense corona but without raised areas called domes, which is the case in the intestine (Urmila *et al.* 2019). According to Neutra *et al.* (1987) the LNs had a germinal centre containing proliferating B-lymphocytes, follicular dendritic cells (FDC) and macrophages. The LN was surrounded by the corona, consisting of small lymphocytes expressing IgM and IgD; the dome presented in intestine lied above the follicle and contained B- and T-lymphocytes, dendritic cells (DCs) and macrophages (Neutra *et al.*, 1987). Urmila *et al.* (2019) reported that the LNs in duodenum, jejunum and ileum showed outer darkly stained area called cortex and inner lightly stained region called medulla. The presence of germinal centres also was reported by Kapoor & Singh (2015) in the LNs of buffalo calves. According to some authors in domestic animals, the epithelium over the dome region was devoid of villi and goblet cells (Dellmann & Eurell, 1998; Kapoor & Singh, 2015; Urmila *et al.*, 2019). The absence of goblet cells in the domes is in accordance with the findings of Medina (1981) in calves and pigs, as well as those of Shuchi & Singh (1996) in dogs. In our study, due to the deeply situated LNs, domes were not observed and single goblet cells were defined in the glandular epithelium near the corona of LNs. Like in submucosa of duodenum, jejunum and ileum (Urmila *et al.*, 2019) it was found out that LNs in bile ducts were encapsulated by connective tissue capsule which was made up of collagen and elastic fibres. Also, it was detected that like in porcine intestine (Urmila *et al.*, 2019) wavy elastic fibres were localised around the follicles in porcine extrahepatic bile ducts. According to Yasuda *et al.* (2004) the germinal centres developed only in bovine jejunal Peyer's patches and colonic lymphoid tissue, but not in the ileal Peyer's patches.

The lymphoid tissue in bile ducts had primary and secondary LNs separated by interfollicular areas. The DLT was obvious in the *lamina propria* at all studied ages, and LNs were also added from two months of age and their thickness increased significantly with age. For the first time, the current study provided data regarding the presense and size of ALNs in porcine extrahepatic bile ducts. ALNs in the terminal segment of DCH, similar to the PPs in porcine intestine (Urmila *et al.*, 2019), were separated into three main domains: the follicular area, the interfollicular area and the nodule-associated epithelium. LGCs were observed in TM mainly and consisted of well-defined LNs and internodular lymphoid tissue penetrated by branching extensions of mucosal glands. ALT of the bile ducts showed similar characteristics to that of the intestine, described by others (Eurell & Frappier, 2006; Urmila *et al.*, 2019), containing LNs, an internodular region rich in lymphocytes (according to Eurell & Frappier, 2006 these were T cells), postcapillary venules through which lymphocytes recirculate and a nodule-associated epithelium. According to Urmila *et al.* (2019) the nodule-associated epithelium covering the dome lacked goblet cells but included

M cells with numerous microfolds on their luminal surface. M cells typically enfolded groups of lymphocytes and occasionally surrounded macrophages and dendritic cells (Eurell & Frappier, 2006). In contrast, the current study showed the presence of single goblet cells as component of nodule-associated epithelium in bile ducts in agreement with the study of Chu *et al.* (1979).

Porcine intestinal LGC was described by Morfitt & Pohlentz (1989). Distribution, histologic structure, and epithelial ultrastructure of colonic LGCs were determined in 5- to 13-week-old pigs. LGCs consisted of well-defined submucosal masses composed of LNs and internodular lymphoid tissue penetrated by radially branching extensions of mucosal glands which corresponds to findings of our study on bile duct LGC. In the study of Urmila *et al.* (2019), the LGCs were noted in the lamina propria and submucosa of the colon (and in the lamina propria of rectum). These LGCs consisted mainly of lymphatic tissue with numerous lymphocytes and glands of lamina propria and submucosa. That is why the LGCs were subdivided into two types i.e. superficial and deep type. The superficial type was present in lamina propria whereas the deep type was present in colonic submucosa. Submucosal LGCs were not however observed in the rectum (Kapoor & Singh, 2015; Urmila *et al.*, 2019). LGCs may also be involved in defence mechanism of the gut in addition to their normal secretory activity. Similarly, Dev Choudhury *et al.* (2017) noted LGCs in the *tela submucosa* of distal caecum, colon and rectum of pigs. LGC in bile ducts was represented by LNs and biliary glands localised in muscular layer of DC, DHC and DCH (except the terminal segment of the extrahepatic part of DCH) belonging to the deep type. In the terminal segment of the extrahepatic part of DCH and PDM, LGC were localised in subepithelial and subglandular connective tissue layer of lamina propria so that they belonged to superficial and deep types.

In intestine, LGC epithelium contained goblet cells, cuboidal and columnar enterocytes, enteroendocrine cells, individual and clustered intraepithelial leukocytes, and cells morphologically compatible with follicle-associated epithelial cells/M cells (Morfitt & Pohlentz, 1989). The present histological study showed that LGC epithelium in porcine bile ducts was consisted of goblet cells, glandular secretory cells, individual and clustered intraepithelial leukocytes. Morfitt & Pohlentz (1989) regard the colonic LGC as a distinct mucosal lymphoid organ and suggest a significant role for it in local and systemic immune responses. According to Morfitt & Pohlentz (1989), the porcine colonic LGC may serve as a model for the human LGC.

It was found that in bile ducts, the LNs were localised in TM and arranged in one to three rows according to type of bile ducts and the age of animals. For example in DC, LNs were arranged in one row, in DHC – in one row, in DCH – in one to three rows, and in PDM – in two rows. According to Urmila *et al.* (2019) the LNs in the submucosa of porcine duodenum were arranged in a single row, but in jejunum and ileum: into two irregular rows. Kapoor & Singh (2015) also noted LNs in multiple layers in the ileum of buffalo calves, which is similar to the study of Urmila *et al.* (2019), whereas Po *et al.* (2005) noted 3–4 rows of LNs in the jejunum and ileum of calves.

In porcine bile ducts, the shape (predominantly oval, some of them round and piriform) of LNs was similar to that in

porcine intestine and dependent of the type of bile ducts. The height and width of LNs also depended on the bile duct types and the age of pigs and were similar to those of porcine intestine. For example, Urmila *et al.* (2019) reported that in pigs, the submucosa of duodenum showed oval shaped LNs of different size with mean LNs height and width 369.09±14.26 μm and 397.66±16.57 μm, respectively. The presence of lymphoid follicles in duodenum was a characteristic feature of pis. Similarly, Abe & Ito (1977) described LNs in the duodenum of mouse.

The shape of the LNs in porcine jejunum varied from oval, elliptical to piriform. Their height was approximately 366.92 ± 23.32 μm and width 399.23 ± 31.11 μm. The mean height and width of LNs in ileum was 440.90 ± 36.72 μm and 417.72 ± 32.37 μm, respectively. These findings suggested that in the ileum the follicle size was greater than the size of LNs in the duodenum and jejunum. Further, the density of LNs was greater in ileum than in the other parts of small intestine (Urmila *et al.*, 2019). In view of the above, in ileum the Peyer's patches appeared as a continuous band like structure. Medina (1981) reported cylindrical to egg-shaped LNs in calves and pigs. Urmila *et al.* (2019) observed the aggregation of the spherical LNs in the submucosa of the ileocaecal junction. The height and width of LNs was 329.71±18.39 μm and 311.71±18 μm, respectively. In middle and distal colon and rectum, the lymphoid element was composed of one or more elliptical submucosal LNs. In colon, the height of LNs was 247.50±20.42 μm and the width of the LNs – 349.37±32.2 μm. In rectum, the height of the LNs was 238.18±28.62 μm and the width was 261.81±44.98 μm.

In ileum, the Peyer's patches (PP), were distinct and oval to elliptical in shape and separated from each other by fibrous interfollicular tissue (Medina, 1981). In the current study, ALNs were identified only in the initial and terminal part of DC in pigs at 6 months of the age but no ALNs were observed in 2-month- and 3-year-old animals. ALNs were localised in TM of the duct. In 6-month-old animals, the diameter of ALNs in the initial part was bigger than in the terminal part of the cystic duct ($P<0.0001$).

The present study showed that follicle (nodule)-associated epithelium (FAE) in porcine bile ducts consists of single goblet cells, glandular secretory cells, individual and clustered intraepithelial leukocytes. Therefore it is different from the intestinal FAE consisting of a single cell-thick layer composed of enterocytes and specialised epithelial cells termed M-cells (Owen & Jones, 1974). The FAE overlies the PP and forms the interface between the intestinal lymphoid system and the intestinal luminal environment (Urmila *et al.*, 2019). Like in intestine, the present study has found a distinct inter follicular region between the LNs in DC, DHC and initial and middle segments of extrahepatic part of DCH. However, in the terminal segment of extrahepatic part of DCH, intramural part of DCH and in the PDM, the interfollicular region was narrow. According to Urmila *et al.* (2019) these interfollicular regions consisted of large number of collagen and reticular fibres, connective tissue cells, blood vessels and nerves. In porcine bile ducts, the elements of internodular regions were similar but elastic fibres were detected in addition. In the duodenum and jejunum, the interfollicular areas were wide whereas in ileum, the interfollicular regions were narrow in size.

CONCLUSION

The current study provides original information about the presence of extrahepatic bile duct associated lymphatic tissue (EHBDALT) as another component of MALT in domestic pigs. The existence of well-developed lymphoid tissue represented by diffuse lymphoid tissue, lymphoid follicles and lymphoid aggregates in extrahepatic bile ducts indicates its role in body defense mechanism, involved in local and systemic immune responses together with the well-known components of MALT in gastrointestinal, respiratory and reproductive tracts.

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REFERENCES

Abe, K. & T. Ito, 1977. A qualitative and quantitative morphologic study of Peyer's patches of the mouse. *Archives of Histology and Cytology*, **40**, 407–420.

Brandtzaeg, P. & R. Pabst, 2004. Let's go mucosal: Communication on slippery ground. *Trends in Immunology,* **25**, 570–577.

Campbell, D. J. & E.C. Butcher, 2002. Rapid acquisition of tissue-specific homing phenotypes by CD4+ T cells activated in cutaneous or mucosal lymphoid tissues. *Journal of Experimental Medicine*, **195**, 135–141.

Chu, R. M., R. D. Glock, R. F. Rossand & D. E. Cox, 1979. Lymphoid tissues of the small intestine of swine from birth to one month of age. *American Journal of Veterinary Research*, **40**, 1713–1719.

Dellmann, H. D. & J. Eurell, 1998. Textbook of Veterinary Histology, 5th edn, Lippincott Williams and Wilkins, Baltimore, pp. 137–191.

Dev Choudhury, K. B., M. Sarma, M. Talukdar, C. K. Gautam, N. N. Barman & A. Deka, 2017. Anatomy of solitary lymphoid nodules in large intestine of post weaned pigs. *International Journal of Chemical Studies*, **5**, 510–512.

Eurell, J. A. & B. L. Frappier, 2006. Dellmann's Textbook of Veterinary Histology, 6th edn, Blackwell Publishing Professional, Ames, Iowa, USA, 139–143.

Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, C. Kato & S-Y. Song, 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity*, **21**, 527–538.

Kapoor, K. & O. Singh, 2015. Ileal and jejunal Peyer's patches in buffalo calves: Histomorphological comparision. *Veterinary World*, **8**, 1273–1278.

Lalitha, P. S., 1991. Gut-associated lymphoid tissue in Indian buffaloes (*Bubalus bubalis*). *Indian Veterinary Journal*, **68**, 1057–1059.

Liebler-Tenorio, E. M. & R. Pabst, 2006. MALT structure and function in farm animals. *Veterinary Research*, **37**, 257–280.

Medina, A. T., 1981. Morphologic characteristics of the epithelial surface of the aggregated lymphoid follicles (Peyer's patches) in the small intestine of newborn gnotobiotic calves and pigs. *American Journal of Veterinary Research*, **42**, 232–236.

Morfitt, D. C. & J. F. L. Pohlenz, 1989. Porcine colonic lymphoglandular complex: Distribution, structure, and epithelium. *American Journal of Anatomy,* **184**, 41–51.

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

Nomina Anatomica Veterinaria, 2017. Sixth edition (revised version). Prepared by the

International Committee on Veterinary Gross Anatomical Nomenclature (I.C.V.G.A.N.) and published by the Editorial Committee Hanover (Germany), Ghent (Belgium), Columbia, MO (U.S.A.), Rio de Janeiro (Brazil), pp. 30, 31, 51.

Owen, R. L. & A. L. Jones, 1974. Epithelial cell specialization within human Peyer's patches: An ultrastructural study of intestinal lymphoid follicles. *Gastroenterology*, **66**, 189–203.

Pabst, R., 2020. The pig as a model for immunology research. *Cell and Tissue Research*, **380**, 287–304.

Po, S. P., A. B. Z. Zuki, M. Zamri-Saad, A. Rahman-Omar & A.W. Effendy, 2005. Morphological study of the jejunal and ileal Peyer's patches of three-month old calves. *Journal of Animal and Veterinary Advances*, **4**, 579–589.

Shuchi, S., & G. K. Singh, 1996. Histological studies on the gut associated lymphoid tissue (GALT) during postnatal development in dogs. *Indian Journal of Veterinary Anatomy,* **1**, 27–30.

Urmila, T. S, P. J. Ramayya, M. S. Lakshmi & A. V. N. Siva Kumar, 2019. Histomorphological studies on gut associated lymphoid tissue of pig (*Sus scrofa*). *The Pharma Innovation*, **8**, 97–101.

Whitmore, I., 1999. Terminologia Anatomica: New terminology for the new anatomist. *Anatomical Record*, **257**, 50–53.

Yasuda, M., M. Fujino, T. Nasu & T. Murakami, 2004. Histological studies on the ontogeny of bovine gut-associated lymphoid tissue: Appearance of T cells and development of IgG+ and IgA+ cells in lymphoid follicles. *Developmental and Comparative Immun*ology, **28**, 357–369.

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