ONTOGENY OF JEJUNAL PEYER'S PATCHES IN INDIAN BUFFALO: A HISTOMORPHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

The histomorphological studies on ontogeny of Peyer's patches in jejunum of 20 buffalo fetuses ranging from 11.5 cm CVRL (80 days) to 100 cm CVRL (299 days) were conducted. The fetuses were categorized into three groups based on their curved crown rump length (CVRL). The fetuses of gestational age from 11.5 cm CVRL (80 days) to 28 cm CVRL (136 days) were devoid of typical jejunal lymphoid patches. However, at 32 cm CVRL (145 days) aggregates of 2 to 3 lymphocytes were observed arranged linearly in submucosa of jejunum Elongated to oval lymphocytic aggregations were observed in submucosa at 54 cm CVRL (195 days) that formed primordia of lymphoid follicle. The presence of darkly stained lymphocytes was also observed in the intestinal villi above the aggregates at this stage. At 70 cm CVRL (231 days), a number of round, pear shaped developing lymphoid follicles were encountered. At 100 cm CVRL (full term), completely developed lymphoid follicles of different shapes i.e., oval, pear shaped and square shaped follicles were present that were arranged in a single row on the anti-mesenteric part of jejunum. The dome of lymphoid follicle was completely formed at this age by invading the jejunal villi that formed arcs over it. Therefore, the present study suggests that the jejunal Peyer's patches started its development at mid-gestational age and become completely developed in the fetuses that reached upto full term just before birth. Thus these jejunal Peyer's patches generates immune response by sampling foreign antigens entering the lumen and play a crucial role in terms of generating mucosal immunity.

Keywords: buffalo, *Bubalus bubalis*, ontogeny, jejunum, peyer's patches, lymphoid tissue

INTRODUCTION

A variety of pathogens enter an animal's body orally and to provide protection to body, oral immunity is provided by the lymphoid tissue present in gut. This is known as Gut associated lymphoid tissue (GALT). Gut, therefore, acts as first line of defense towards antigens entering through oral route. GALT is present in ileum and jejunum as the Peyer's patches (PP) and thus plays a critical role in immune defence against antigens inserted to the alimentary tube. These Peyer's patches are the origin of lymphocytes migrating to the mucosal sites of action, thus providing

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immunity. Peyer's patches (PP) are therefore the most frequently studied structures in the gut-associated lymphoid tissues forming a central part of the inductive site of the mucosal immune system (Brandtzaeg and Pabst, 2004). Moreover, the ileal Peyer's patches has been extensively studied in sheep, cattle, pigs, rodents and man (Cornes, 1965; Faulk *et al*., 1970; Sobhon, 1971; Owen and Jones, 1974; Chu *et al*., 1979; Sminia *et al*., 1983; Reynolds and Morris, 1983; Liebler, 1985). Moreover, the prenatal development of ileal peyer's patches has been explored in bovines (Beyaz and Asti, 2004) and in buffalo (Kritima and Singh, 2015). But embryological development of jejunal peyer's patches especially in buffalo has received very less attention. Therefore, the present work was undertaken to study the embryological development of jejunal peyer's patches in buffalo.

MATERIALS AND METHODS

Collection of samples

The current study was conducted on jejunum of buffalo fetuses (n=20) of different gestation age varying from 11.5 cm CVRL (80 days) to 100 cm CVRL (299 days) collected from different abattoirs. Instantly after collection, their curved crown rump length (CVRL) in centimeters was measured and their approximate age of fetuses was calculated by formula given by Soliman (1975):

$$Y = 28.66 + 4.496\ X \quad (CVRL < 20\ cm)$$

$$Y = 73.544 + 2.256\ X \quad (CVRL \geq 20\ cm)$$

Where Y is age in days and X is curved crown rump length (CVRL) in cm.

Depending upon CVRL, fetuses were divided into three groups i.e., Group I: CVRL between 0 to 20 cm (0 to 118 days), Group II: CVRL between 20 to 40 cm (119 to 163 days) and Group III: CVRL > 40 cm (164 to 310 days).

Histological staining

Tissue pieces from jejunum were fixed in 10% neutral buffered formalin and processed for paraffin sectioning by dehydrating in ascending grades of alcohols and acetone, cleared in benzene and infiltrated in paraffin (Luna, 1968). Sections of 4 to 5 µ thickness were stained with haematoxylin and eosin for routine histology (Luna, 1968), Masson's trichome for collagen fibres (Luna, 1968), Gridley's for reticular fibres and Verhoeff's for elastic fibres (Sheehan and Hrapchak, 1973).

Immunohistochemical staining

The paraffin sections of 4 to 5 µm in duplicate were mounted on super frost positively charged slides (Fisher Scientific). After deparaffinisation and rehydration, heat induced antigen retrieval was done in citrate buffer (AR 3 solution, Biogenex) and heating in microwave at 95°C for 10 minutes and 98°C for 5 minutes. Sections were washed in 0.1 M phosphate buffered saline (pH 7.4). The endogenous peroxidase activity was blocked by immersing the sections in 3% (v/v) H_2O_2 in methanol for 20 minutes followed by washing in 0.1 M phosphate buffered saline (pH 7.4). To prevent non specific binding of antibodies sections were blocked with normal horse serum (Vector's Laboratories USA). The sections were incubated with primary antibody (Anti CD_3^+ - Pan T cell marker) at 4°C for overnight in humidified staining box. After washing in 0.1 M phosphate buffered saline (pH 7.4), the sections were incubated with universal secondary antibody (Vector Laboratories, USA). The chromogen used

was 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories, USA) and sections were counterstained with Gill's III haematoxylin. The sections were washed in running tap water, dehydrated, cleared and mounted with DPX.

RESULTS AND DISCUSSION

In buffalo fetuses of gestational age from 11.5 cm CVRL (80 days) to 28 cm CVRL (136 days) no typical jejunal lymphoid patches were observed. However, at 32 cm CVRL (145 days) aggregates of 3 to 4 lymphocytes were observed that were arranged linearly in submucosa of jejunum on the anti-mesenteric side (Figure 1). Yasuda *et al.* (2006) described that in sheep and cattle, the prenatal development of follicles in the Peyer's patch began first in the jejunum during the middle of gestation. These submucosal aggregates were confirmed to be lymphocytic aggregates by localizing them with anti CD_3^+ antibody and they were found to be mainly CD_3^+ T lymphocytes (Figure 2). This anti CD_3^+ antibody marks pro-thymocytes, the stem cells from which T-cells arise in the thymus and that is initially expressed in the cytoplasm of pro-thymocytes. They differentiate into common thymocytes, then into medullary thymocytes, and at this latter stage, CD3 antigen begins to migrate to the cell membrane. The antigen is then found to be bound to the membranes of all mature T-cells (Chetty and Gatter, 1994). The anti CD_3^+ antibody acts as Pan T-cell marker and therefore, immunohistochemically marked the T lymphocytes that migrated from thymus to the submucosa of jejunum via high endothelial venules (HEV) to initiate the development of lymphoid patches (Binns and Pabst, 1994). The intraepithelial lymphocytes (IELs) were also observed at this age (Figure 2).

At 35 cm CVRL (152 days), these intraepithelial lymphocytes were observed to increase within the developing villous epithelium at this stage (Figure 3). These intraepithelial lymphocytes (IELs) were observed to be CD_3^+ T lymphocytes present in the embryologically differentiating villous epithelium of jejunum (Figure 4). Montilla *et al.* (2004) also observed that almost all IELs are CD3+ lymphocytes and these intraepithelial cells are functionally heterogeneous population that contains cytotoxic T lymphocytes with anti-tumor activity and natural killer activity. It is likely that antigens crossing the epithelium get processed and presented to these T lymphocytes to generate mucosal immune response.

With the advancing gestational age, at 54 cm CVRL (195 days) larger elliptical lymphoid aggregates were observed in submucosa on the anti-mesenteric side that formed the primordia of lymphoid follicles. Just above the lymphocytic aggregations that formed primordial lymphoid follicle, the villous epithelium also had lymphocytes that tend to form dome shaped structure above it (Figure 5). Similar observation was found in ileum of buffalo fetuses by Kapoor and Singh, 2015; Beyaz and Asti, 2004 in ileum of bovine fetuses. This finding was confirmed by localization of CD_3^+ T lymphocytes at similar locations i.e., in lymphoid primordia and within the dome above it.

However, at 70 cm CVRL (231 days), lymphoid follicles were observed to have started developing into small and compact asymmetrical follicles on anti-mesenteric side. Particularly these follicles were arranged in a single row in the submucosa of jejunum on the anti-mesenteric side. Moreover, at this stage interfollicular spaces had started to develop around the compact lymphoid follicles. Also at places, diffuse lymphoid tissue

(DLT) was also observed to have started developing within the interfollicular spaces in-between the lymphoid follicles, completely surrounding the lymphoid follicles at some places at this age (Figure 6). Each follicle was surrounded by abundant collagen and reticular fibers in its thin developing capsule around it. Also few reticular fibers were observed in the center that supported the cellular framework of lymphoid follicle (Figure 7).

At 80cm CVRL (254 days), the interfollicular spaces were observed to be wider, completely surrounding the lymphoid follicle, a feature that is particular to jejunal lymphoid tissue, found in neonates after birth as well (Kritima and Singh, 2015). The diffuse lymphoid tissue has also become wider at this age. At some places, along with other developing lymphoid follicles, this diffuse lymphoid tissue was also observed to invade the alternating villi to form an arc shaped area in between villi known as dome. The lamina muscularis mucosae was disrupted at the area of invasion of dome in between villi (Figure 8). Kikukawa *et al.* (2012) also observed that the fetal stage also had jejunal Peyer's Patches composed of lymphatic follicles, the interfollicular region, and the dome region in lesser mouse deer. The epithelium lining the dome was more eosinophilic as compared to adjacent villi and was lined by nearly cuboidal epithelium. The rest of the lymphoid follicle below dome was composed of lighter area in the centre that formed germinal centre (GC) that has started differentiating slightly at this age. Further, the similiar differentiation into a light central zone and a darker periphery was observed in ileum of sheep fetuses at 130 to 135 days of gestation (Nicander *et al*., 1991) and in bovine fetuses at 271 days of gestation (Beyaz and Asti, 2004).

At 100 cm CVRL (299 days), the lymphoid follicles of varying shapes (elliptical or oval, rectangular to square) and size were observed to be arranged in a single row on the anti-mesenteric side (Figure 9). The lymphoid follicles at this age had more prominent dome that invaded in between villi and the dome was lined by columnar epithelium with intraepithelial cells at this stage as well (Figure 12 and Figure 13). Here, also the follicles were covered by a capsule composed of both collagen and reticular fibers. The apex of the lymphoid follicles had thin capsule whereas the tapering base of lymphoid follicles at some places had thick collagen fibers that extended these collagen fibers into submucosa, joining the submucosal connective tissue. This submucosal connective tissue framework had network of lymph vessels and high endothelial venules (HEV) (Figure 10).

The parenchyma or the center of the lymphoid follicles contain framework of collagen and reticular fibers that developed structural framework (Figure 11). However, the parenchyma of the lymphoid follicle is composed mainly of lymphocytes i.e., B cells and T cells, plasma cells, macrophages and follicular dendritic cells (FDC).

The interfollicular spaces became completely wide at this age and were occupied by lymph vessels that helped in migration of lymphocytes and thus generation of immune response. The diffuse lymphoid tissue present in interfollicular region was quite evidently wider at this age and was also composed of prominent blood capillaries and high endothelial venules (HEV) (Figure 12 and Figure 13). At this age also, there was slight differentiation of lymphoid follicle into dark outer region called cortex and light inner region called germinal center (GC).

The outer dark cortex was mainly composed of CD_3^+ T lymphocytes. Also, the

interfollicular region at this stage, i.e., formed by the diffuse lymphoid tissue comprised CD_3^+ T lymphocytes (Figure 14). Similar observations were made by Kikukawa *et al.* (2012) in lesser mouse deer. However, few round lymphoid nodules were observed to be present above the intact lamina muscularis mucosae intermixed with villous epithelium and thus formed a structure known as propria nodules (PN) (Figure 12 and Figure 15). Similar propria nodules were reported by Leibler *et al.* (1988) in large intestine of calves.

CONCLUSION

The present study revealed that jejunal peyer's patches started developing prenatally in buffalo fetuses from mid-gestational period and got completely developed into lymphoid patches in full term fetuses. Therefore, jejunal lymphoid peyer's patches developed before birth during prenatal life. Moreover, these prenatally developed jejunal peyer's patches continue to exist in the adult life and generate mucosal immune response by producing antibodies in the form of immunoglobulins to protect the body from antigens entering the gut. Therefore, this characteristic of jejunal lymphoid tissue i.e., to generate mucosal immune response can be exploited further for developing oral vaccines in large animals also like buffaloes.

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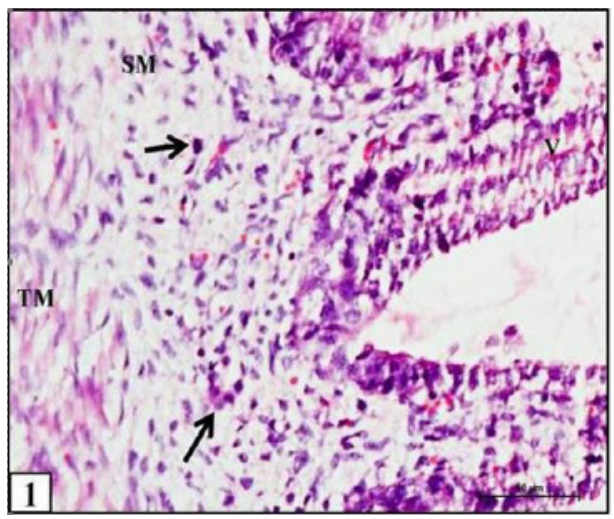

Figure 1. Photomicrograph of jejunum of 32 cm CVRL (145 days) fetus showing group of lymphocytes (arrows) at scattered locations in submucosa (SM), villi (V) and tunica muscularis (TM). H and E X400.

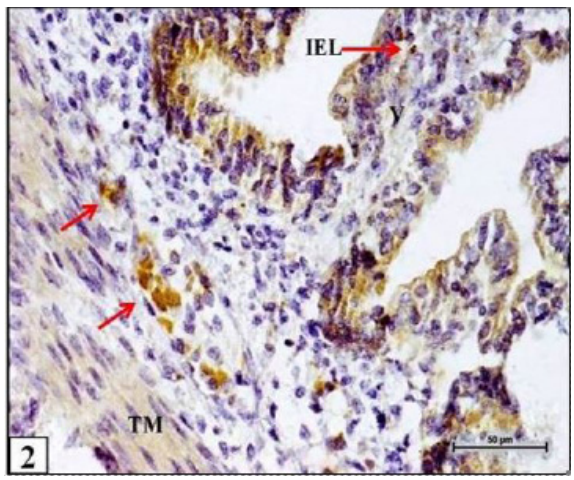

Figure 2. Immunostained sections showing CD_3^+ T lymphocytes (arrows) in submucosa and villi (V) with IEL (intraepithelial lymphocytes) in same age fetus. HRP X400.

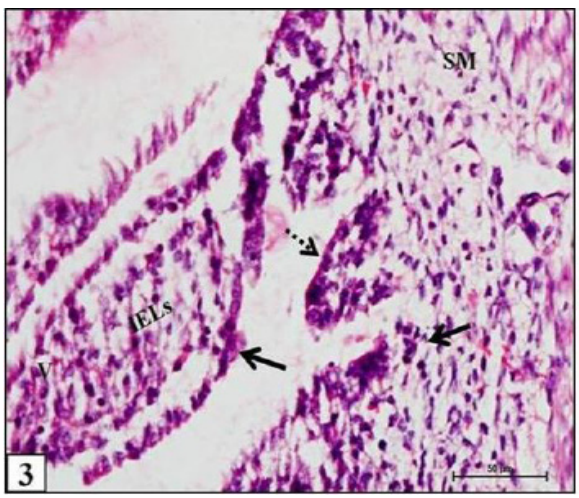

Figure 3. Photomicrograph of 35 cm CVRL (152 days) fetus showing presence of submucosal (SM) lymphoid aggregates (small arrow), intra epithelial lymphocytes (IELs, dotted arrow) and tunica muscularis (TM). H and E X400.

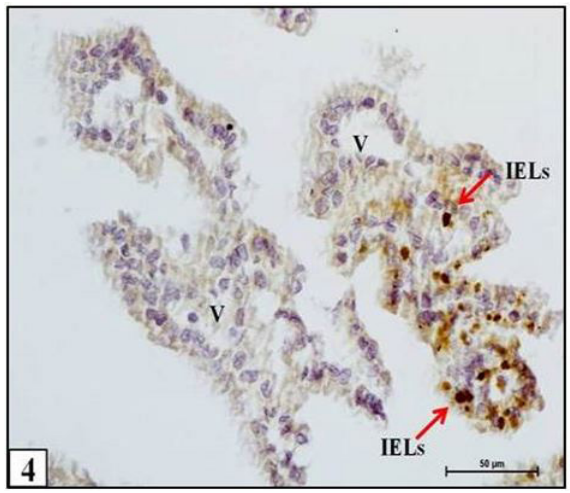

Figure 4. Same age immunostained sections showing CD_3^+ T lymphocytes in villous epithelium (V) as intra epithelial lymphocytes (IELs). HRP X400.

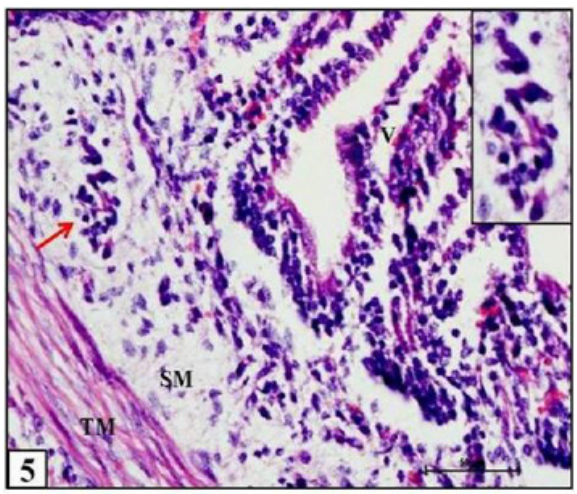

Figure 5. Photomicrograph of 54cm CVRL (195 days) fetus showing larger elliptical lymphoid aggregates (arrow) in submucosa (SM) on the anti-mesenteric side that formed the primordia of lymphoid follicles and tunica muscularis (TM). H and E X100. [Inset: Lymphocytic aggregate].

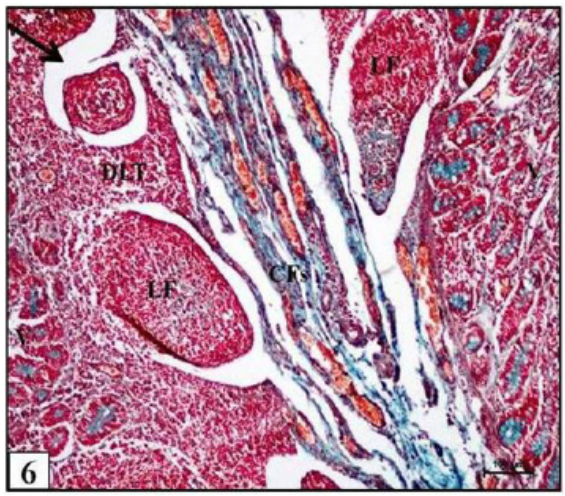

Figure 6. Photomicrograph of 70 cm CVRL (231 days) fetus showing small, compact asymmetrical lymphoid follicles (LF) with interfollicular spaces (arrow), diffuse lymphoid tissue (DLT) in between and collagen fibers (CFs). Masson's Trichrome X100.

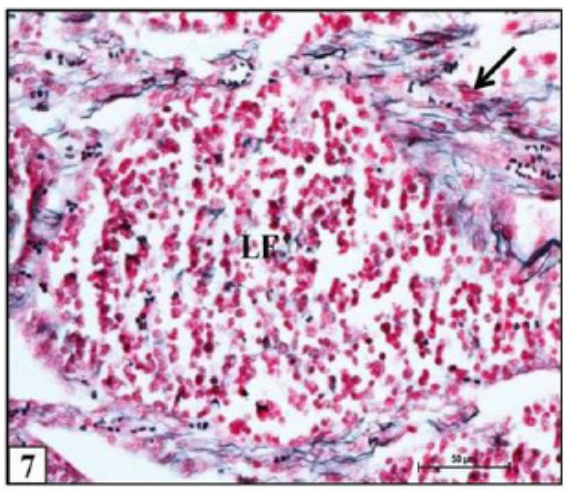

Figure 7. Photomicrograph of 70 cm CVRL (231 days) fetus showing a lymphoid follicle (LF) with distribution of lymphocytes and other immune cells embedded in reticular fiber framework and the capsule containing reticular fibers. Gridley's X400.

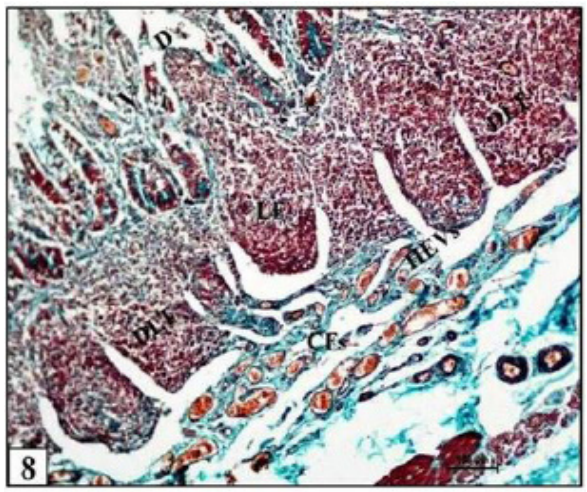

Figure 8. Photomicrograph of 80 cm CVRL (254 days) fetus showing dome (D) in between villi, wide interfollicular spaces, diffuse lymphoid tissue (DLT) and abundant high endothelial venules (HEVs) intermingled in collagen fibers (CFs) in submucosa. Masson's Trichrome X100.

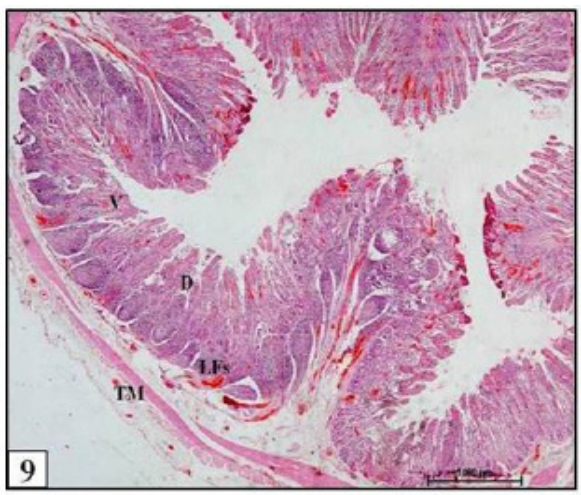

Figure 9. Photomicrograph of 100 cm CVRL (299 days) fetus showing presence of oval elliptical lymphoid follicles (LFs) arranged in a single layer on the anti-mesenteric side, dome (D) in between villi (V) and tunica muscularis (TM). H and E X40.

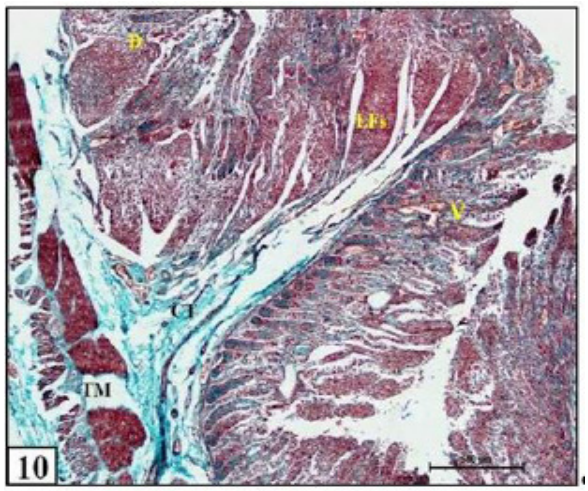

Figure 10. Photomicrograph of same age fetus showing lymphoid follicles (LFs) covered by a collagen capsule that extended in submucosa as connective tissue strands (CT). Masson's Trichrome X100.

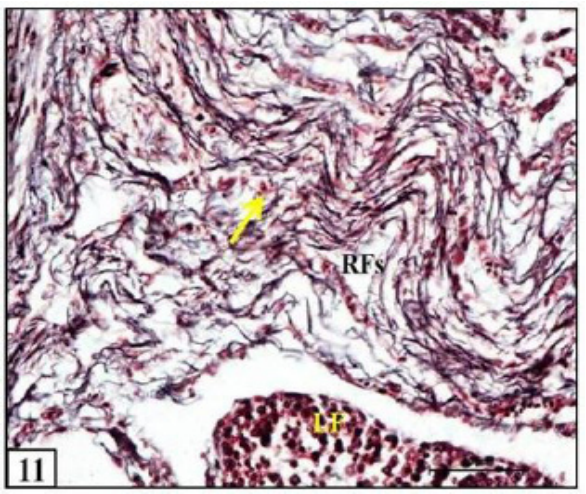

Figure 11. Photomicrograph of 100 cm CVRL (299 days) fetus showing presence reticular fiber framework in parenchyma of lymphoid follicles (LFs) and interfollicular connective tissue (arrow). Gridley's X400.

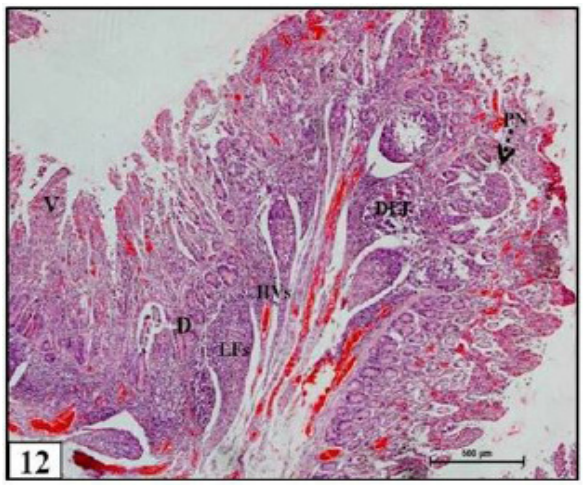

Figure 12. Photomicrograph of same age fetus showing lymphoid follicles (LFs), diffuse lymphoid tissue (DLT), high endothelial venules (HEVs) and propria nodules (PN, dotted arrow). H and E X40.

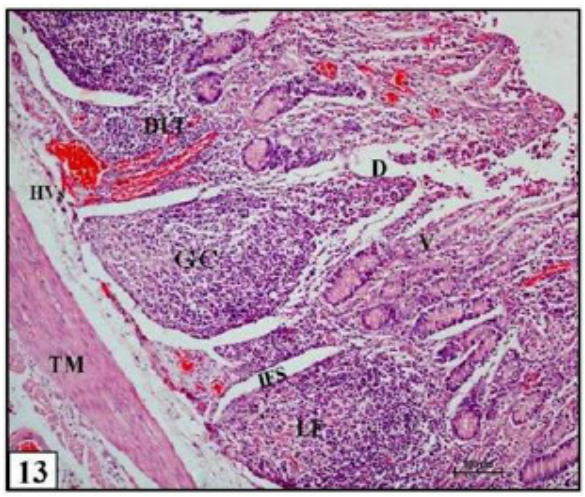

Figure 13. Photomicrograph of 100cm CVRL (299 days) fetus showing lymphoid follicle (LF) protruding in villous (V) epithelium forming prominent dome (D), germinal center (GC), large interfollicular diffuse lymphoid tissue (DLT), high endothelial venules (HVs) and tunica muscularis (TM). H and E X100.

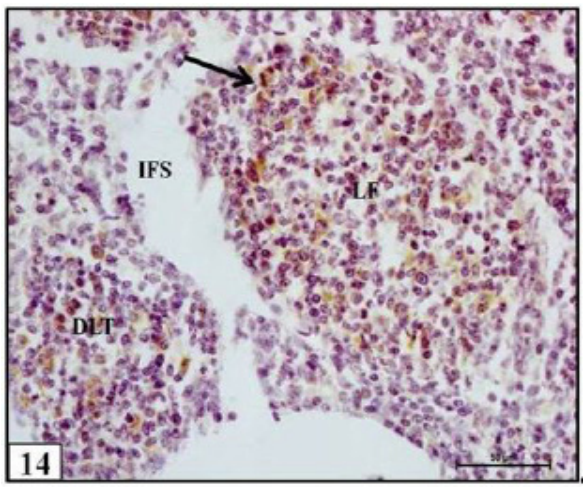

Figure 14. Immunostained sections of same age showing mainly CD_3^+ T lymphocytes (arrows) in outer dark cortex (arrow) of lymphoid follicle and in center of diffuse lymphoid tissue with wide interfollicular space (IFS). HRP X400.

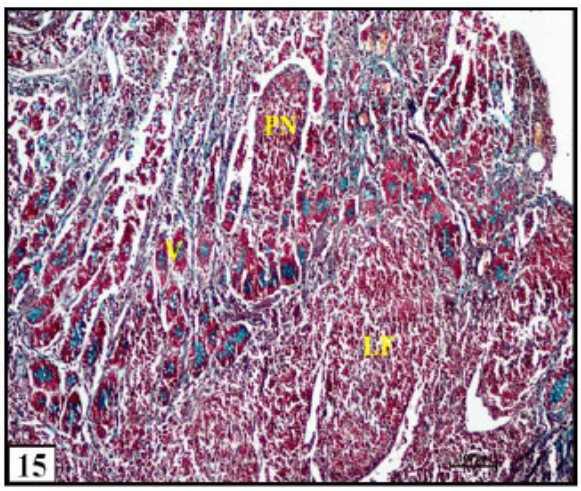

Figure 15. Photomicrograph of 100 cm CVRL (299 days) fetus showing few lymphoid nodules above the intact lamina muscularis mucosae within the villous epithelium (V) that formed propria nodules (PN). Masson's Trichrome X100.

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