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Gross and histological study on nasopharyngeal tonsil of dogs (*Canis familiaris*)

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Abstract
The present research work is carried to know the distribution and nature of lymphoid tissue in the nasopharyngeal tonsil of adult dogs. The materials of the study was collected in eight adult dogs which were euthanized in renal failure cases across Bangalore city. The heads were dissected to locate the pharyngeal tonsil by cutting the head into two half by band saw machine. One half is fixed in 2% acetic acid solution, the other half is used to study the gross parameters and samples were collected for histology in different fixatives. Grossly, pharyngeal tonsil is appeared as thin sheet of raised mass of organised lymphoid tissue located on the posterior wall of the nasopharynx and distal to the opening of auditory tube. Histologically, pharyngeal tonsil is lined by pseudo stratified ciliated columnar epithelium with basal supporting cells and few goblet cells. The Follicle Associated Epithelium (FAE) which interrupts the continuity of epithelia at places are the migrations of lymphocytes from the follicles into the epithelium which is also known as reticular epithelium which is lined by simple to stratified cuboidal cells with less number of collagen and reticular fibre below the basement membrane.

Keywords: Pharyngeal tonsil, dogs, fixatives, microfold cells, epithelium

Introduction
The mucous membrane lining the digestive and the respiratory systems are continuously exposed to various pathogens, pollens, and dust particles in the environment, to which the body must either react to produce an immune mediated response or it should maintain immunological tolerance (Hiller *et al*. 1998; Debertin *et al*. 2003; Simecka, 1998) [16, 14, 33]. Most of the antigens that tackle the innate immunity and enter through special type of cell called Microfold cell (M cells) which allows the antigen to enter the epithelial layer, wherein it will be encountered by the mucosal immune system.
The special kind of immune system that is developed on the mucosal layer of the different systems of the body called as the mucosal immune system. The Mucosa Associated Lymphoid Tissue (MALT) which represents a first line of defence system plays an important role in initiating the immune response at the entry level of the pathogen. Mucosal immune system contribute about 80% of the immune cells which are accumulated at various MALT; which are distributed in mucosal surfaces, is an essential part of the mucosal immune system.
M-cells are specialized cells lining within epithelia which act as the gateway for antigens to the immune system. Following endocytosis of antigen, vesicular transcytosis occurs through the M-cell cytoplasm and the antigen is presented to dendritic cells, macrophages, T lymphocyte and B lymphocytes (Miniggio, 2016) [26]. MALT contains lymphatics which transport immune cells and antigens to regional lymph nodes that can therefore be called part of the inductive sites of mucosa and augment the immune responses (Liebler-Tenorio and Pabst, 2006) [20].
Tonsils are secondary lymphoid organs which consist of aggregations of lymphoid cells located in the oro-pharynx, nasopharynx and laryngopharynx of most species except rodents (Cocquyt *et al.* 2005) [11]. Tonsils (naso and oro tonsils) together form a ring of lymphoid tissue in the pharyngeal wall called the "Waldeyer ring" (Perry and Whyte 1998) [29]. Von Waldeyer-Hartz, first described a 'ring' of lymphoid tissue in the human pharynx, in 1884. 'Waldeyer's ring' consists of different tonsils like nasopharyngeal, tubal, palatine, para-epiglottic, lingual tonsils and tonsil of soft palate with species variations. These tonsils represent large aggregates of mucosal lymphoid tissue located in the pharynx.
Scientists are working on the future route of vaccination; mainly by nasal and oral route of vaccination which provides a practical alternative to intra muscular or intra dermal

vaccination as these routes of administration will be directly targeting the induction site for strong mucosal immune responses. This induces a combination of local and systemic responses.

The nasopharyngeal tonsil is the main targets for nasal vaccines in case of dog. Presently kennel cough vaccines are available in the market and it's been used widely against *Bordetella bronchiseptica* by intra nasal route.

The dog being a very friendly and obedient pet animal which is gaining popularity in this century is utmost importance in the studies regarding immune system as there are chances of getting many zoonotic diseases. Although extensive works have been done on the tonsils in other animals, a comprehensive study on the canine nasopharyngeal tonsils is needed. A detailed study on the different cells of the canine tonsils would be highly useful for researchers to develop different kind of vaccine. This concept of vaccination is based on the use of ligands that selectively bind to M cell surface carbohydrates, meaning that coating the vaccine antigens with the appropriate ligands will enhance vaccine delivery (Casteleyn *et al*. 2013) [8].

Hence the aim of the present study is

1. Investigation of gross anatomical location and gross morphology of the nasopharyngeal tonsil in dogs (*Canis Familiaris*).
2. To study the histological characteristics of the nasopharyngeal tonsil in dogs (*Canis Familiaris*).

Materials and Methods

Tissues were collected from eight adult dogs from post mortem of either sex without any breed specifications and of different age which were euthanized (with intravenous overdose of Thiopentone sodium) due to renal failure around Bangalore city. The dogs had no history of respiratory diseases and were not treated with any corticosteroids as they were suffering from renal failure.

The sagittal sections of the heads were taken using Bone and Meat cutting machine and were washed thoroughly in tap water and the macroscopic appearance like color, size, shape, location, extent, morphometry and topographic relation of nasopharyngeal tonsil were recorded.

To observe clearly the nasopharyngeal tonsil, remove the mandible (lower jaw) and soft palate was opened longitudinally and the head was kept in a solution containing 2% acetic acid solution for 4 hrs to observe the lymphatic nodules (Cornes, 1965; Chauhan And Singh, 1970) [12, 10]. This method of fixation is done as the nasopharyngeal tonsils of dogs are not well appreciated on a naked eye.

Tonsils were dissected and fixed in the fixatives like 10% neutral buffered formalin, Bouins fluid and Zenker's fixative for histological studies.

After fixation in the appropriative fixatives for minimum 48 hours the samples were cut to required thickness 1 cm cube and orientations, later washed in tap water to remove the excess fixative. Then the tissues were processed for the histological studies following the techniques of Luna (1968) [23] by dehydrating in ascending grades of alcohol (50%, 70%, 80%, 90% and 3 changes of absolute alcohol) for 3hrs each. Then the tissues were cleared with 3 changes of chloroform for 3 hours each. The tissues were impregnated by liquid paraffin with ceresin overnight and later on with 2 changes of liquid paraffin with ceresin for 3 hours each and mounted on tissue embedding cups to make paraffin blocks. Sections of 4µm thickness were cut using microtome and stained with routine Haematoxylin and Eosin stain (Luna, 1968) [23] for histology study. The stained tissue sections were observed under light microscope (Olympus BX53) linked to a digital camera (Magcam- 5). Special staining procedure were also done for collagen fibre by Masson's trichrome method (Luna, 1968) [23], demonstration of Reticular fibre by Gomori's method for reticulum (Luna, 1968) [23], Toulidine blue (Luna, 1968) [23] and PAS stain (Luna, 1968) [23] for demonstration of mucosubstances.

Results and Discussion Gross observations: In the present study the nasopharyngeal tonsil in dog is an organised raised mass of lymphatic tissue which is located on the posterior wall of the roof of nasopharynx just caudal to nasal septum and distal to the opening of auditory tube (Fig. 1). It extends as a thin sheet towards the oropharynx and the tonsil does not have any folds or crypts like in other species. The nasopharyngeal tonsil was found well developed in many species like goat, pig, cat, and bovine however in dog nasopharyngeal tonsil is not well delineated and macroscopically hardly visible similar to the horse (Casteleyn *et al*. 2011) [6]

When the tonsil is removed it appears as pinkish red thin sheet of tissue that's been attached to the bone. The epithelium is coated by a layer of mucus which acts as innate immunity preventing the entry of pathogens. On an average the tonsil length was 20 mm and width was about 7.6 mm.

The nasopharyngeal tonsil exposed in the head appeared as a small white patch confirming the lymphatic nodules after fixation with 2% acetic acid for a day (Fig. 2).

The position of the tonsil activates the immune system in sampling of antigen passing through oro and naso pharynx at the level of entry of pathogens similar to the observations made by of Kumar and Timoney (2001) [19] in horse and Liu *et al*. (2012) in pigs.

Cesta (2006) [9] explained that there are two types of tonsils, namely; tonsils with crypts (follicular tonsils) and those without crypts. The crypts are are blind, often branched invaginations of surface epithelium into the submucosal lymphoid tissue. Tonsils without crypts typically have a slight folded surface epithelium (Banks, 1993) [1]. In the present study, grossly there was absence of crypts on the surface of nasopharyngeal tonsil which was similar to the observations made by Billen *et al*. (2006) [4] where he noted that the nasopharyngeal tonsil of dogs was oval shaped pale pink to red area which generally appeared as a flattened plaque, sometimes elevated with an irregular micronodular surface and no crypts or folds were present in it. This might be due well developed turbinates and the absence of sweat glands in the skin which makes the dog to breathe more through the mouth, compared to other species of animals which breathe mainly through nose. This alteration makes less exposure of the pathogens to the nasopharyngeal tonsils in dogs so the tonsil is not well appreciated as in other animals.

Many bacterial and viral pathogens like the canine parvo virus, canine distemper virus, infectious canine hepatitis virus, Bordetella bronchoseptica, and tuberculosis which affect the dog when they sniff the infected fomites. So this study will give anatomical detail of the nasopharyngeal tonsils in dogs. Miniggio (2016) [26] and Casteleyn *et al.* (2007) [7] explained that the presence of crypts will increase the epithelial surface area there by if any pathogens enter the crypt there will be

more time for the pathogen to interact with the epithelial surface which facilitates the uptake of foreign antigens entering the nasopharynx during breathing.

Histological observations: Nasopharyngeal tonsillar epithelium

The nasopharyngeal tonsillar mucosa in dogs is lined by pseudostratified ciliated columnar epithelium (Fig. 4) with many goblet cells in the present which was similar to the earlier reports of Kumar *et al.* (2001) [19] in equines. The basal cells were present at the base of the epithelium and the nucleus was basophilic and oval shape. Transitional to squamous type of epithelium was observed in the caudo-ventral area of the tonsil (Fig. 5) which was similar to the observations made by Billen (2006) [4] in dogs. Both the epithelium and the underlying dense connective tissue were infiltrated by lymphoid cells which were similar to the reports of Liu *et al.* (2012).

The epithelium was modified irregularly at places into Follicle Associated Epithelium (FAE) wherein the pseudo stratified ciliated columnar epithelium is modified into simple to stratified cuboidal cells this region is also known as reticular epithelium which is characterized by low height of cells with less number of nuclie, absence of ciliated cells and been infiltrated with lymphocytes (Fig. 3 & Fig. 4). The continuity of epithelial basement membrane will break at lymphothelium with penetrating lymphocytes from the follicles (Fig. 3, 4 & 6) and the goblet cells were absent at these area of epithelium. Kumar *et al*. (2011) [16] suggested that in sheep nasopharyngeal tonsils these lymphocytes originated from the underlying lamina propria. Whereas Mair *et al*. (1987) [25] opined that the topography of FAE above lymphoid nodules was the basis that they act as a mechanism for trapping and sampling antigens in the airstream.

In the present study the intraepithelial lymphocytes increased towards the base of the FAE and these lymphocytes which are migrated from the follicles are penetrating between the epithelium giving disruption in the continuity of epithelium, with few reticular fibres and few collagen fibres just below the lymphothelium and the lymphoid nodules (Fig. 6 & 7). Mabbott *et al.* (2013) [24] described FAE as a single layer of epithelial cells which was similar to the observations made by Corr *et al.* (2008) [13], these epithelium overlies lymphoid tissue of the inductive sites characterised by special type of cells known as M cells (Bockman and Cooper, 1973) [5], these cells plays a key role in generating mucosal immune response as described by Spit *et al.* (1989) [34] and they acts as a transporter of antigen across epithelial barrier (Sicinski *et al.* 1990; Neutra, 1999; Hathaway and Kraehenbuhl, 2000). Toppets *et al*. (2011) [27, 32, 36] suggested that the massive intraepithelial lymphocyte infiltration suggested that the nasopharyngeal tonsils were perfectly adapted to sample foreign antigens.

There are two types of epithelium lining the tonsils namely the reticular and non-reticular epithelium. The reticular epithelium which is spongy in nature and located at the apices of lymphoid follicles, (Fig. 7) and it's been supported by the reports of Belz and Heath (1995) [2] in dogs; Cesta (2006) [9]. It contains different cells like M cells, dendritic cells, lymphocytes, macrophages (Bernstein, 1999) [33]. The non-reticular epithelium, which separates reticular epithelium, is lined by psuedostratified ciliated columnar epithelium (Fig 2), which was clearly observed in the present study and it is similar to the observations made by Kumar *et al.* (2017) [17] in pigs.

The presence of special type of cells called as microfold cells (M cell) can be appreciated in the epithelium between the ciliated columnar cells (Fig. 10) and seen more in the region of lymphothelium. These M cells were having microfold instead of cilia at the surface and stained less when compared with adjacent cells. The M cells will be usually associated with intraepithelial lymphocyte which pushes the nucleus towards the base of the cell (Fig. 10). These cells act as a transporter of pathogens/microbes from the epithelial barrier into the system was similar to the observations made by Kumar *et al* (2001) [19] in equines.

The mucus on the surface of the nasopharyngeal eoithelium of dogs and the goblet cells in the epithelium were showing strong PAS reaction (Fig. 8) except at the region of lymphothelium. The absence of mucus and goblet cells on the lymphothelium indicate that it would be expected to favour the direct contact of antigens similar to the observations made by Kumar *et al.* (2001) [19] in equines. The lack of goblet cells in the FAE reduced the thickness of the epithelium (Owen *et al.* 1986) [28] and modified the composition of mucus layer over FAE. Lloyd Mayer (2003) [22] explained that the mucin glycoprotein that is lining the surface epithelium is produced by the goblet cells which act as a barrier covering adjacent epithelium which traps the bacteria, virus and other dust particles which gets expelled from the body. The mucin produced by the goblet cells also serves as a reservoir for secretory IgA.

Stanley *et al.* (2001) [35] explained that the close contact and communication between the epithelium and lymphocytes in the pockets of microvillous cells are crucid to their role in antigen uptake and processing. These observations confirmed the earlier descriptions given by Kumar and Timoney (2001) [19] in equines; he opined that the occurrence of intraepithelial lymphocytes was a regular feature of the nasopharyngeal tonsil of the horse which was similar to the reports of (Miniggio, 2016) [26].

In the present study, the nasopharyngeal tonsil of dogs both nasal and oral types of epithelium i.e., pseudostratified ciliated columnar, transitional and stratifed squamous epithelium lined the nasopharyngeal tonsils since it extended up to the intrapharyngeal opening of nasal cavity, which was similar to the observation made Billen *et al*. (2006) [4] in dogs.

Tonsil Proper

The connective tissue just below the epithelia was made up of mainly by collagen than of reticular fibres, but the thickness of the collagen layer decreases towards the reticular epithelium. A thin layer of collagenous connective tissue which separates the epithelium from the lamina propria above the submucosa. The lamina propria is made of mainly collagenous type of connective tissue which supports the aggregates of lymphoid tissue, fine blood capillaries, few nerve fibres and lymphatics to drain the lymph. The collagen and reticular fibres were sparsely distributed in the sub epithelial area and dense in deeper parts (Fig. 6 & 7). In the submucosa clusters of glandular acini (mixed type of gland) were separated from the lymphoid tissue by loose irregular connective tissue (Fig. 6) and few acini in glands shows strong PAS positive reaction indicating presence of neutral mucosaccharides (Fig. 9). The glands were surrounded by collagen (Fig. 6) and can observe the distribution of high

endothelial venules (HEV) in the parafollicular regions.

The lymphoid tissue of the nasopharyngeal tonsil was mainly composed of diffuse lymphatic nodular area and large number of lymphocytes which are scattered freely all along the submucosa (Fig. 3) which was similar to the observations made by Liebler-Tenorio and Pabst (2006) [20] in other domestic species. The diffuse lymphatic tissue was composed of small, medium and large lymphocytes, plasma cells, mast cells, macrophages, dendritic cells and fibroblasts. The lymphoid nodules were surrounded by a parafollicular and interfollicular area composed of a thin meshwork of reticular fibres and a few collagen fibre. Scadding (1990) [31] explained that the tonsils containing T cells will be involved in the immunological function either by promoting it as helper T cells or by preventing it by suppressor T cells. Whereas Ranjit *et al.* (2016) [30] explained that the lymphoid tissue was separated from the glandular tissue by collagen, elastic and reticular fibres in pigs.

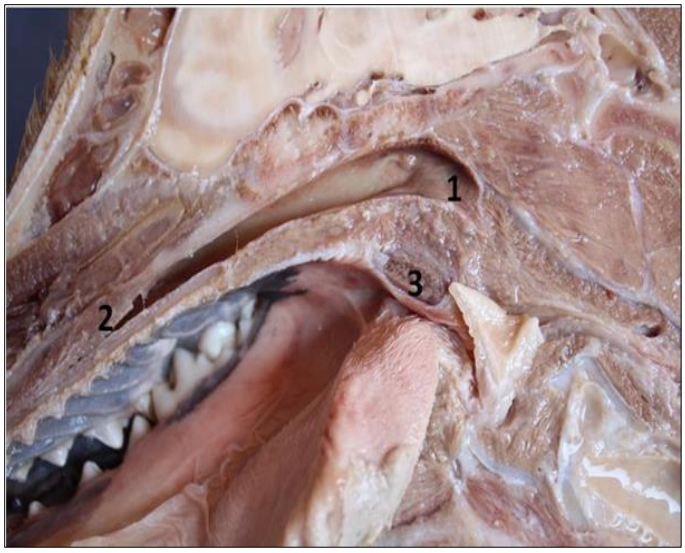

Fig 1: Gross photograph of sagittal section of head of the dog showing location of nasopharyngeal tonsil (1), nasal septum (2) and palatine tonsil (3)

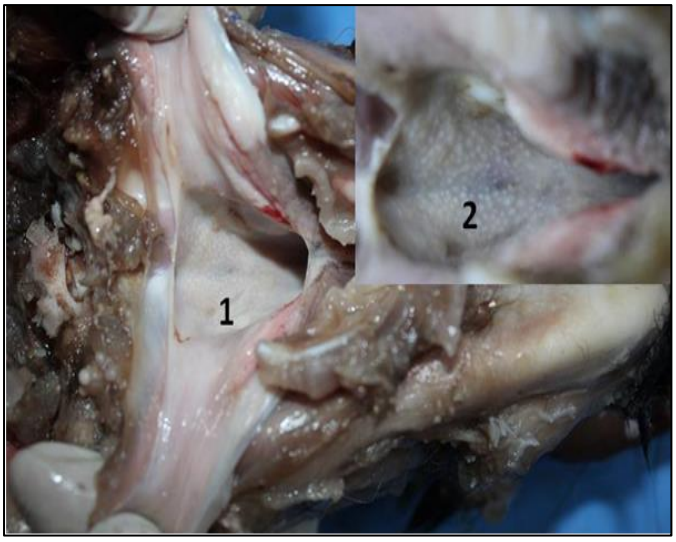

Fig 2: Gross photograph showing nasopharyngeal tonsil of dog (1) after fixation of specimen with 2% acetic acid. The presence of white patches in the nasopharyngeal tonsil (2) indicating the presence of lymphoid follicles.

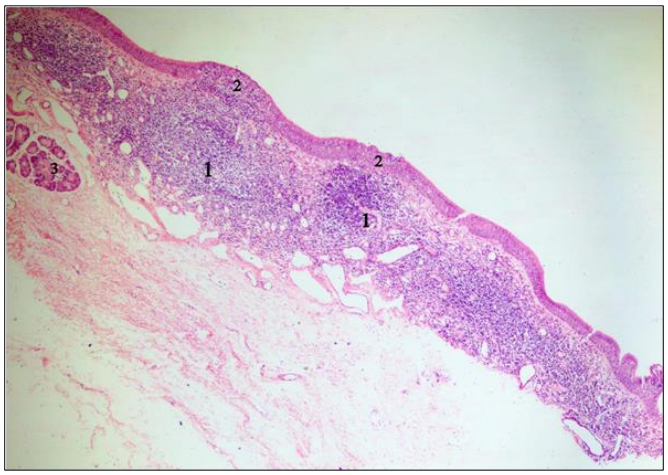

Fig 3: Photomicrograph of nasopharyngeal tonsil of dog showing lymphothelium (2), lymphoid follicle (1) and the sub mucosal gland. H&E X 40

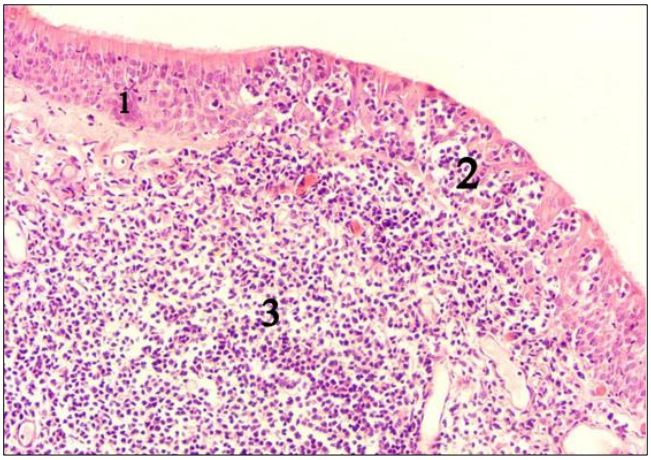

Fig 4: Photomicrograph Pharyngeal tonsil of dog showing pseudostratified ciliated columnar epithelium (1) and the lymphothelium (2) where in the lymphoid cells penetrating the epithelium from the follicle (3). H&E X 200

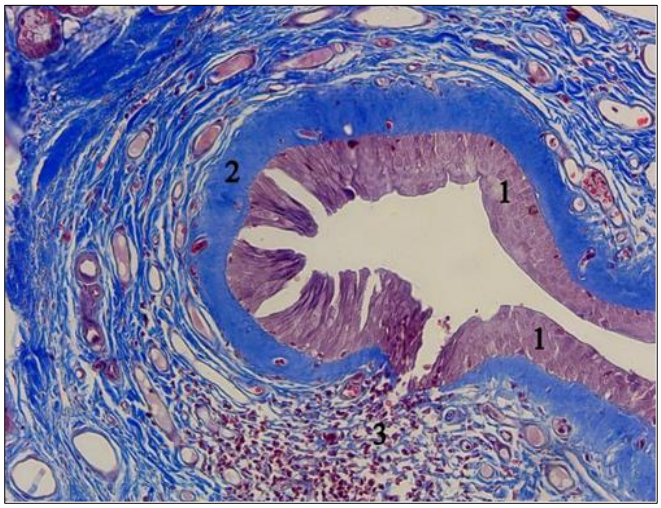

Fig 5: Photomicrograph showing psuedostratified ciliated columnar epithelium that is lining the dog's nasopharyngeal tonsil is transforming into stratified squamous type of epithelium (1) at the end of tonsil where the nasal side is folding to oro-pharynx. The thick layer of collagen layer is present just below the epithelia (2) and can observe the lymphoid cells penetrating the epithelia from the nodule (3). Masson's Trichrome X 200

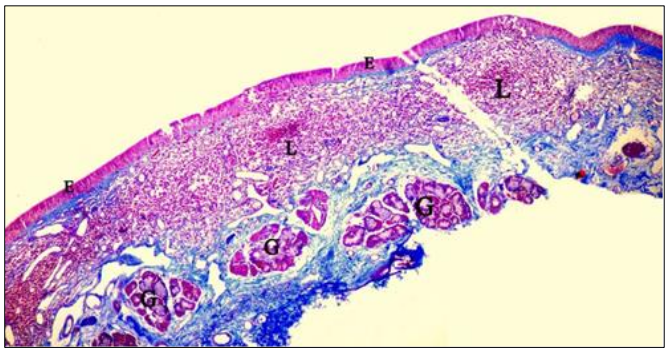

Fig 6: Photomicrograph showing collagen fibre (blue) running below the epithelium (E) showing decreased amount of collagen in lymphoepitheium region. The collagen fibre surrounding the sub mucosal glands (G) and surrounding the lymphoid follicle (L) indicating structural support to the submucosa in the nasopharyngeal tonsil of dogs. Masson's trichrome X 40

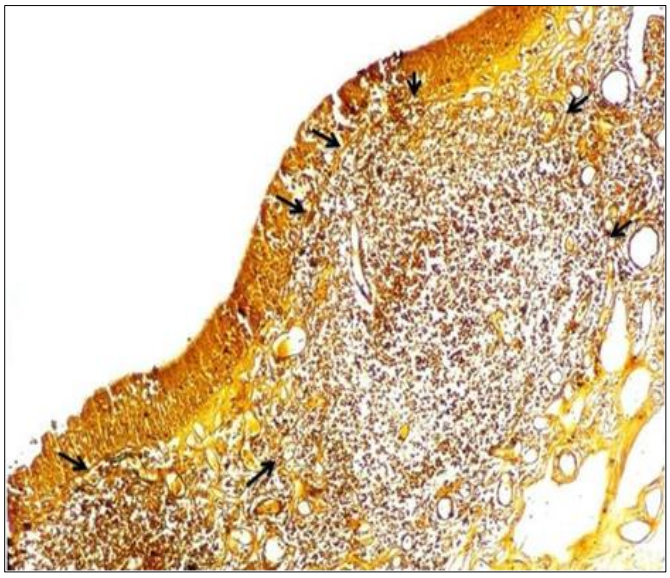

Fig 7: Photomicrograph showing a thin layer of reticular fibre (black arrows) lining just below the pseudo stratified ciliated columnar layer and at the region of lymphothelium, the fibres also surrounding the lymphatic follicle in the nasopharyngeal tonsil of dogs. Gomori's X 100

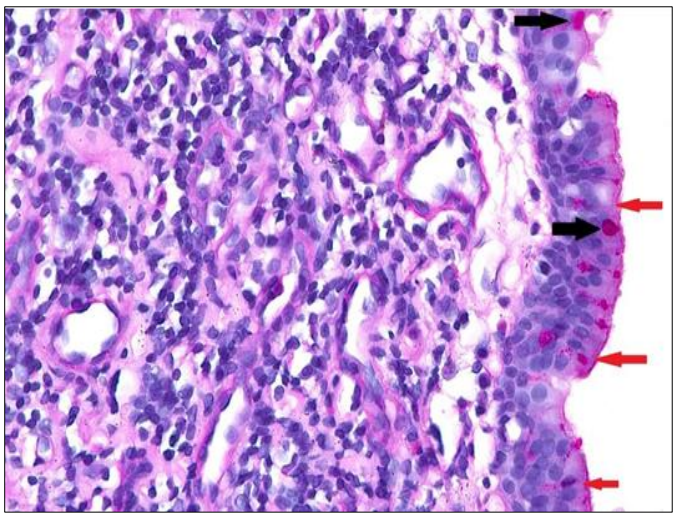

Fig 8: Photomicrograph showing Goblet cells (black arrows) showing strong PAS reaction in the epithelium. The mucus on the surface of epithelia (red arrow) also showing strong PAS reaction in nasopharyngeal tonsil of dog. PAS X 200

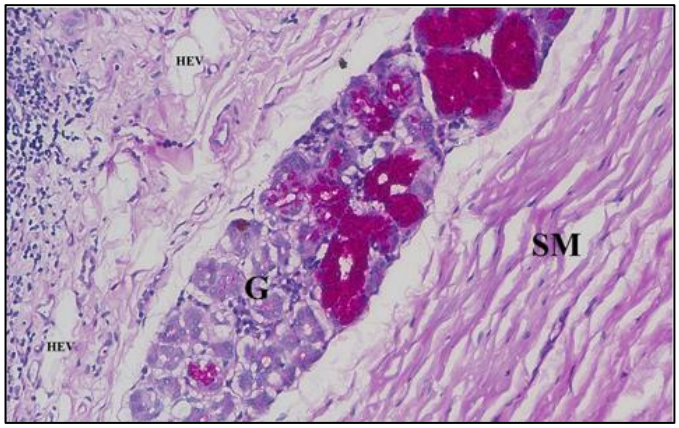

Fig 9: Photomicrograph showing the mixed Acini (glands) (G) with strong PAS reaction in the sub mucosa (SM) with high endothelial Venule's (HEV) of pharyngeal tonsil in dogs. PAS X 200

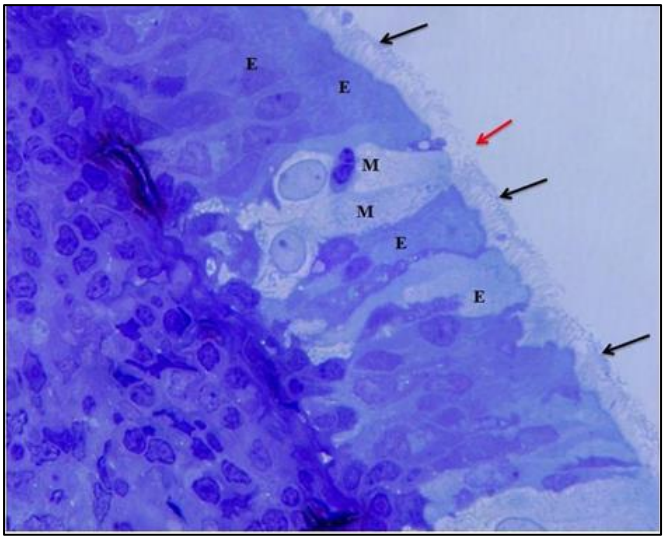

Fig 10: Photomicrograph showing M cell (M) no cilia (red arrow showing microfold on the surface of M cell) and it is lightly stained compared to adjacent epithelia (E). The nucleus located at the basal region and also presence of lymphocyte indicate it is M cell. The psuedostratified columnar cells (E) with cilia (black arrow) adjacent to M cell in nasopharyngeal tonsil in dogs. Toulidine blue X 200

Conclusion

The nasopharyngeal tonsil in dog is being located at the roof of the nasopharynx. It serve as a primary antigen sampling point which encounter most of the antigens entering through the nasal cavity at the route of entry and present them to the APCs and initiate an immune response. This mechanism of sampling antigen will help in development of nasal vaccines in dogs for various infectious diseases.

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