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Prashanth Kumar KS
Ph.D. Scholar, Department of Veterinary Anatomy and Histology, Veterinary College, KVAFSU, Bangalore, Karnataka, India

Ashok Pawar
Professor and Head, Department of Veterinary Anatomy and Histology, Veterinary College, KVAFSU, Bidar, Karnataka, India

Girish MH
Associate Professor (i/c) and Head, Department of Veterinary Anatomy and Histology, Veterinary College, KVAFSU, Bangalore, Karnataka, India

Ganganaik S
Professor and Head, Department of Veterinary Anatomy and Histology, Veterinary College, KVAFSU, Gadag, Karnataka, India

Suguna Rao
Professor and Head, Department of Veterinary Pathology, Veterinary College, KVAFSU, Hebbal, Bangalore, Karnataka, India

Jagadish Hiremath
Senior Scientist, National Institute of Veterinary Epidemiology and Disease Informatics, Yelahanka, Bengaluru, Karnataka, India

Rashmi Santhoshkumar
Senior Scientific Assistant, Electron Microscopy- Common Research Facility, Department of Neuropathology, NIMHANS, Bengaluru, Karnataka, India

Corresponding Author:
Prashanth Kumar KS
Ph.D. Scholar, Department of Veterinary Anatomy and Histology, Veterinary College, Hebbal, KVAFSU, Bangalore, Karnataka, India

Gross, histology and ultrastructural study of nasopharyngeal tonsil of pig

Prashanth Kumar KS, Ashok Pawar, Girish MH, Ganganaik S, Suguna Rao, Jagadish Hiremath and Rashmi Santhoshkumar

Abstract
Study was conducted using heads of eight large white Yorkshire pigs procured from the slaughter house. The heads were dissected to locate the nasopharyngeal tonsil. Gross parameters were recorded and samples were collected for histology and ultra-structural studies separately. Grossly, nasopharyngeal tonsil was appearing as a raised mass located on the roof of the nasopharynx in the caudal part of the pharyngeal septum. Histologically, pharyngeal tonsil was covered by pseudo stratified ciliated columnar epithelium with basal supporting cells and goblet cells. This epithelium was modified irregularly at places into Follicle Associated Epithelium (FAE) lined by simple to stratified cuboidal epithelium. In scanning electron microscope, surface of the tonsil was covered with ciliated cells, microvillus cells and in between these Microvillus cells, Microfold cells (M-cells) were observed. Transmission electron microscopy revealed that the M-cells were found all along the epithelium.

Keywords: Tonsils, pigs, microfold cells (M-cells), follicle associated epithelium (FAE)

Introduction
Most of the antigens presented to immune system in a lifetime enter the body through mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts. The mucosal immune system is the part of immune system juxtaposed to the mucosal surface and is composed of the mucosa associated lymphoid tissue (MALT) which represents a first line of defence mechanism.

The German anatomist von Waldeyer-Hartz, commemorated for his coinage of successful medical terms like “chromosome” and “neuron”, first described a ‘ring’ of lymphoid tissue in the human pharynx, in 1884. This ‘ring’ is now termed as ‘Waldeyer’s ring’ and consists of large aggregations of lymphoid nodules in the oropharynx, nasopharynx and laryngopharynx viz., lingual tonsil, palatine tonsil, pharyngeal tonsil, tubal tonsil, paraepiglottic tonsil and the tonsil of the soft palate with species specific variations.

Nasal vaccination now provides a practical alternative to oral vaccination for induction of mucosal immune responses as it induces a combination of systemic and local responses and the adverse reactions are fewer compared to parenteral administration. The pharyngeal and tubal tonsils are the main targets for nasal vaccines. Further, the viruses causing classical swine fever, pseudorabies and foot-and-mouth disease persist asymptomatically within the porcine tonsils and hence they form very important clinical material for diagnostic tests. Therefore, it is necessary to have reliable baseline information of the tonsils in disease free animals.

The pig being a model for most of the biological experiments and a highly prolific animal is of utmost importance in the studies regarding immune system. Although extensive works have been done on the lymphoid organs of pigs, a comprehensive study on the porcine tonsils is scanty. A detailed ultrastructural and immunohistochemical study on the different cells of the porcine tonsils would be highly useful for breeders and researchers. It will form a basis for further studies on their physiology, pathology, immunology and genetics.

Hence a study on gross morphology, histomorphology and ultrastructure of the nasopharyngeal tonsils of pigs (*Sus scrofa domestica*) was envisaged with the following objectives:

1. To study gross and histological characteristics of the nasopharyngeal tonsils of pigs.
2. To study ultrastructural features of the nasopharyngeal tonsils of pig

Materials and Methods

Tissue samples were collected from eight apparently healthy adult pigs of age ranging from seven to nine months, irrespective of sex from the local slaughter house.

The median sections of the heads were taken using bone and meat cutting machine. Both the halves were washed thoroughly in fresh water and the shape, size, colour, location, extent, morphometry and topographic relation of pharyngeal tonsil were recorded. Tonsils were dissected out and fixed in the following fixatives:

1. 10 percent neutral buffered formalin, Bouins fluid, and Zenker's fixative histological studies.
2. 2.5% v/v glutaraldehyde in 0.1 M Phosphate Buffer, at pH 7 for SEM (Shapiro *et al*. 2019) [6].
3. 2.5% v/v glutaraldehyde in 0.1 M Sodium Cacodylate buffer at pH 7.2 for TEM (Shapiro *et al*. 2019) [6].

After fixation in the appropriate fixatives, the tissues were processed for the histological studies following the techniques like Dehydration, clearing, impregnation finally embedding the tissues in paraffin block (Bancroft and Gamble 2003) [1] and Sections of were cut 5-8µm thickness stained with routine Haematoxylin and Eosin stain for histological studies.

Scanning Electron Microscopy (SEM)

Samples of fresh tonsils were evaluated using SEM as described by Shapiro *et al*. (2019) [6] in rabbits. The samples were fixed in 2.5% glutaraldehyde at room temperature for 12 hours. Later the samples were transferred to same fixative for up to 48 h at 4 °C in the followed by washing with 1X PBS twice for 30 min each. Then the samples were postfixed in 1% osmium tetroxide for 2 h at 4 °C which was followed by washing with 1X PBS twice for 30 min each. Later the samples were dehydrated with graded ethanol (20%, 40%, 60%, 80% and 100%) at an interval of 30 min each. Later the samples were subjected to critical point drying for up to 3 h to reach a critical point CO_2 and then CO_2 was bled out. Samples were then placed on the stubs and subjected to gold sputter for 90 seconds so that the gold particles get sputtered over the samples for better visualization under electron beams. Then the samples along with the stub were placed in the SEM chamber (Ziess EVO 18 SEM. Germany) and vacuum was created and the working distance was adjusted manually. The samples were then subjected to electron beams for visualization under monitor at accelerating voltage of 20kV. Finally, images were obtained at different magnifications

Transmission Electron Microscopy (TEM)

For TEM the samples were fixed in 2.5 percent glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) followed by post fixation in one percent aqueous osmium tetroxide. The specimens were dehydrated in ascending grades of ethanol, cleared in propylene oxide and embedded in araldite. Semi-thin sections were cut 1 µm and stained with toluidine blue for orientation and examination. Ultrathin sections 60-80 nm were cut, harvested on carbon-coated 300-wire mesh copper grids, stained with Uranyl acetate and examined at magnification of X 1500 up to X13000 by utilizing TEM at Common research facility, Electron microscope lab, NIMHANS, Bengaluru.

Results and Discussion

Pharyngeal tonsil was a raised mass located on the roof of the nasopharynx in the caudal part of the pharyngeal septum (Fig. 1 and 2.) and had an average of three to four mucosal folds in pigs.

This position facilitated sampling of antigen passing through both respiratory and digestive systems. The observation in the present study is in accordance with the reports of Kumar and Timoney (2001) [15, 22] in horse, Cocquyt *et al*. (2005) [11] and Kumar and Nagpal (2007) [14] in sheep and Liu *et al*. (2012) [24] in pigs. Casteleyn *et al*. (2007) [9] observed that the crypts increased the epithelial surface area of the pharyngeal tonsils and facilitated the uptake of foreign antigens entering the nasopharynx during breathing. In contrast Billen *et al*. (2006) [2] noted that the pharyngeal tonsil of dogs was not easily identifiable and no crypts or folds were present in it. This might be because exposure of the canine nasal and nasopharyngeal mucosa to inhaled antigens was less since the dogs breathed through both their nose and mouth as compared to other domestic animals which breathed mainly through the nose.

Nasopharyngeal tonsils of pig were covered by pseudostratified ciliated columnar epithelium with basal supporting cells and goblet cells (Fig. 3). The basal cells were present at the base of the epithelium with oval, less basophilic nucleus. There were two types of the tall columnar supporting cells based on the staining affinity of their nucleus (Fig. 3). Type-I nuclei were strongly basophilic and mainly placed towards the mid zone of the epithelium. The type-II cells had elongated nuclei with less basophilic chromatin material and were distributed superior to the type-I nuclei. All the cell types presented a finely granular eosinophilic cytoplasm. In between the epithelial cells intraepithelial lymphocytes could be seen at places. These observations confirmed the earlier finding of Kumar and Timoney (2001) [15, 22] in equines, Kumar and Kumar (2005) [12] in goats and Kumar *et al*. (2006) [18, 19] and Kumar *et al*. (2011) [20] in sheep. Kumar and Timoney (2001) [15, 22] opined that the occurrence of intraepithelial lymphocytes was a regular feature of the nasopharynx and nasopharyngeal tonsil of the horse. According to Billen *et al*. (2006) [2] in dogs, both nasal and oral types of epithelia i.e., pseudostratified ciliated columnar, transitional and stratifed squamous epithelium lined the pharyngeal tonsils since it extended up to the intrapharyngeal opening of nasal cavity unlike in pigs where it was positioned on the roof of nasopharynx.

Crypts were formed corresponding to the morphological mucosal folds and were lined by pseudostratified columnar epithelium. This epithelium was modified irregularly at places into FAE lined by simple to stratified cuboidal cells. Its characteristic features were low height, reduced number of rows of nuclei and absence of goblet cells. The observation in the present study is in accordance with the findings of Kumar and Timoney (2001) [15, 22] in horse, Kumar and Kumar (2005) [12] in goats and Kumar and Nagpal (2007) [14] in sheep. The lack of goblet cells in the FAE reduced the thickness of the epithelium (Owen *et al*., 1986) [26] and modified the composition of mucus layer over FAE. The resulting absence of mucus favoured direct contact of microorganisms and their antigens according to Kumar and Timoney (2001) [15, 22] in horse. Mair *et al.* (1987) [25] opined that the topography of FAE above lymphoid nodules was the basis of the suggestion that they acted as mechanisms for trapping and sampling antigens in the airstream. Infiltration of large number of intraepithelial lymphocytes and blood vessels through the disrupted basement membrane were seen in the present study. Kumar and Timoney (2001) [15, 22] also noted free lymphocytes

particularly in large clusters towards the free surface of the epithelium in the crypt areas of the FAE in horse. Kumar *et al*. (2011) [20] suggested that in sheep pharyngeal tonsils these lymphocytes originated from the underlying lamina propria. Toppets *et al*. (2011) [27] suggested that the massive intraepithelial lymphocyte infiltration suggested that the pharyngeal tonsils were perfectly adapted to sample foreign antigens.

Lamina Propria

In pharyngeal tonsils the lymphoid tissue surrounded a central loose collagenous connective tissue core rich in blood vessels and lymph vessels unlike other tonsils. The lamina propria was made of dense connective tissue, dense aggregates of lymphoid tissue, fine blood capillaries and a few nerve fibres. The collagen, reticular and elastic fibres were sparsely distributed in the sub epithelial area and dense in deeper parts. In the lamina propria clusters of glandular acini were separated from the lymphoid tissue by dense collagenous connective tissue. Ducts from the glands opening into the surface epithelium were also seen. Large numbers of HEVs were distributed between the connective tissue in the interfollicular regions. Their numbers were more than that seen in other tonsils.

The lymphoid tissue of the pharyngeal tonsil was mainly composed of diffuse lymphatic tissue in the subepithelial area and large number of small, medium and large lymphatic nodules (Fig. 4) distributed throughout the lamina propria. The diffuse lymphatic tissue was composed of small, medium and large lymphocytes, a few plasma cells, mast cells and macrophages. The lymphoid nodules were surrounded by a parafollicular and interfollicular area composed of a dense meshwork of reticular fibres and a few collagen and elastic fibres. In a few lymphatic nodules dome shaped distinct outer corona formed by large number of small lymphocytes were seen below the follicle associated epithelium. Cryptolymphatic units and tonsillar nodules were also seen.

Scanning electron microscopy of the pharyngeal tonsil revealed a dense mat of ciliated cells (Fig.5) with interspersed microvillus and goblet cells. Majority of the ciliated cells possessed tufts of uniform sized cilia with bulbous endings and these masked the appearance of other cells as reported in horse (Kumar and Timoney, 2005) [16, 17] and sheep (Casteleyn *et al*., 2010; Kumar and Kumar, 2012) [8, . The microvillus cells of different types along with ciliated and brush cells were visible in areas where density of cilia was less (Fig.5). The region of FAE possessed microvillus cells which were categorized into different types depending on the size of microvilli as reported earlier in the horse (Kumar and Timoney, 2005) [16, 17] and in sheep (Casteleyn *et al*., 2010; Kumar and Kumar, 2012) [8, 13]. Type I cells possessed microvilli of uniform size and shape with distinct outlines Type II cells had larger microvilli. Occasional cells with very short microvilli and raised surfaces were identified as M cells (Fig. 6) as reported earlier in the horse (Kumar and Timoney, 2005) [16, 17] and sheep (Kumar and Kumar, 2012) [13].

Transmission electron microscopy illustrated that the epithelial cells were loosely arranged (Fig. 7) and adjacent cells were joined by a number of desmosomes, and with different microvilli size. HEVs in the pharyngeal tonsil were mainly composed of high endothelial cells and contain granulocytes and lymphocytes. Under TEM, various developing stages of lymphocytes with necklace shaped nuclei and other supporting cells were seen in the lamina propria of the tonsils. Few plasma cells (Fig. 9) and macrophages were also noticed. The macrophages had lobular nucleus with peripheral condensation of chromatin and a cytoplasm with lipid bodies, ribosomes and mitochondria. We could observe characteristic M cells which were lacking cilia with a basal nucleus, short microvilli and an intimate association with antigen presenting cells like Dendritic cells, Macrophages and lymphocytes (Fig. 8) as described by Kumar *et al*. (2010) [21] in equines. In the area of lamina propria we could see many plasma cells and other APCs like Macrophages and Dendritic cells.

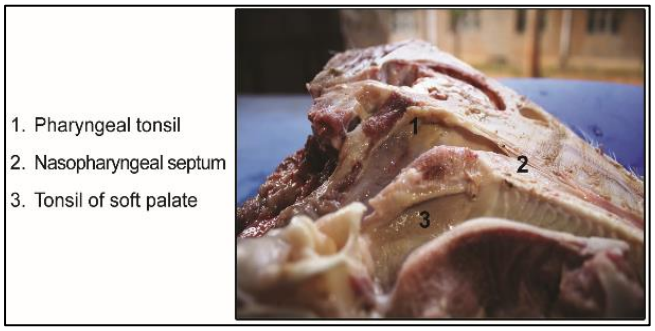

Fig 1: Median view of the nasopharyngeal area of pig showing the location of pharyngeal tonsil

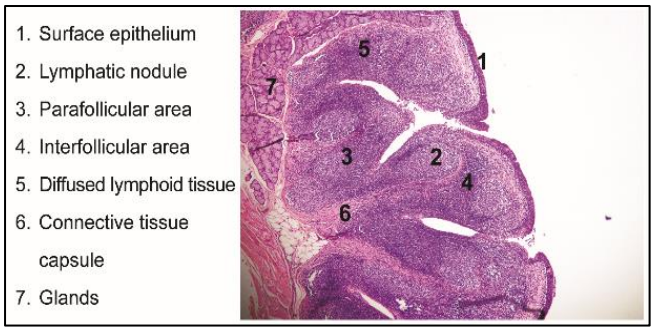

Fig 2: C.S of pharyngeal tonsil of pig. H & E x40

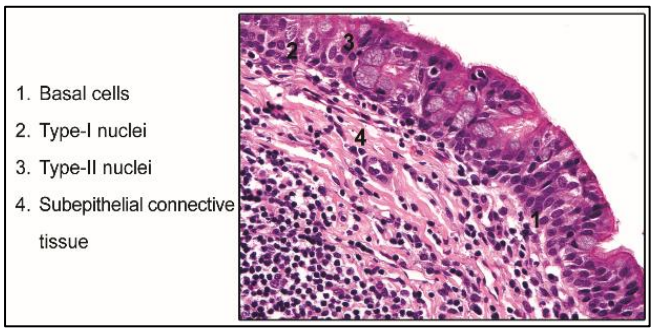

Fig 3: C.S of pharyngeal tonsil of pig showing pseudostratified columnar epithelium. H&E x400

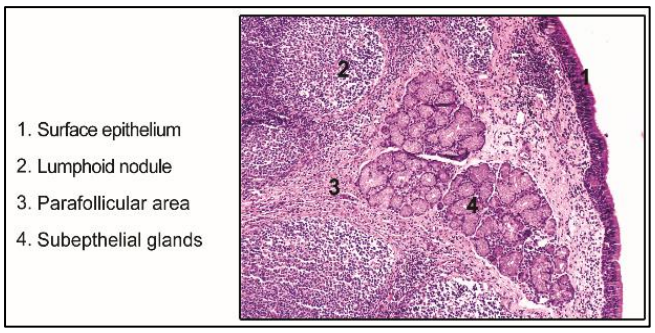

Fig 4: C.S of pharyngeal tonsil of pig. H & E x100

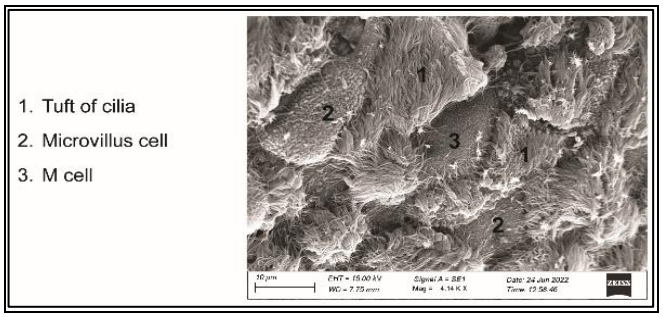

Fig 5: Scanning electron micrograph of pharyngeal tonsil of pig x4100

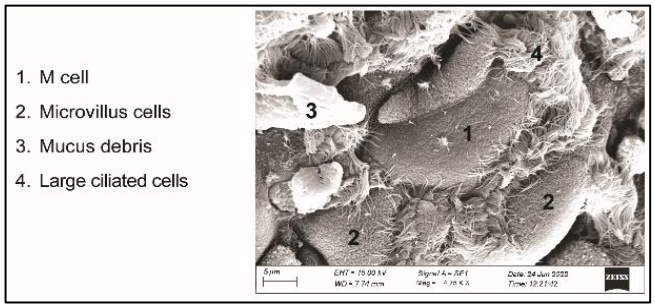

Fig 6: Scanning electron microscope of the pharyngeal tonsil of pig x4750

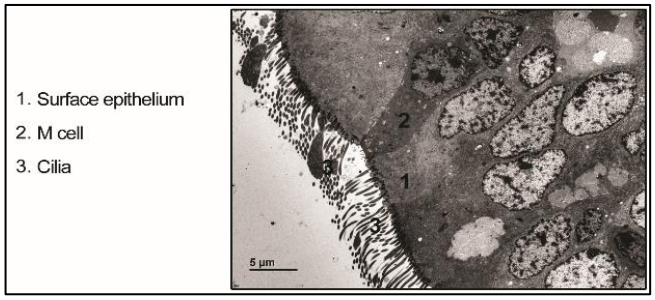

Fig 7: TEM image of surface epithelium with cilia and a nonciliated M cell x600

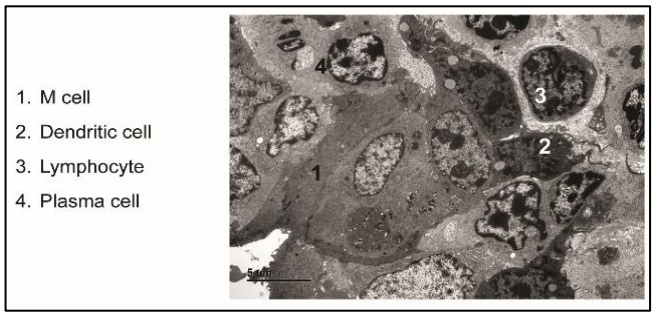

Fig 8: TEM image of M cell with associated dendritic cell and lymphocytes x800

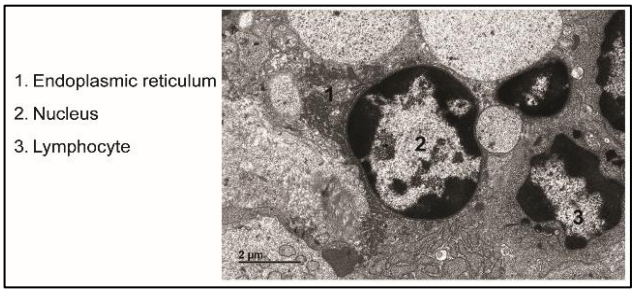

Fig 9: TEM image of Plasma cell in the lamina propria of pharyngeal tonsil of pig

Conclusion

Pharyngeal tonsils in pig being located at the roof of the nasopharynx encounters most of the antigens entering through the nasal cavity and present them to APCs and initiates a strong and rapid immune response. This mechanism of sampling antigen will help in development nasal vaccines in pigs for various infectious diseases. The present study provides the basic structural information for those researches of vaccine development.

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