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Gross morphology, histomorphology and ultrastructural study on lingual tonsil of pig

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Abstract
The present work was carried out to study the gross morphology, histomorphology, histochemistry and ultrastructure of the lingual tonsil in pigs. Tissue samples were collected from eight apparently healthy adult pigs of age ranging from seven to nine months from the local slaughter house. The lingual tonsils of pig were not visible grossly even after the fixation with two percent acetic acid for four hours but in the histological sections, tonsil was seen within the connective tissue cores of the mechanical tonsillar papillae located caudal to the vallate papillae present at the root of the tongue in all the pigs. Histologically, lingual tonsils were lined by a stratified squamous keratinized surface epithelium which is differentiated into stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. The proper tonsillar tissue of tonsils of oropharynx (lingual tonsil and tonsil of soft palate) and laryngopharynx (Paraepiglottic tonsil) were characterized by lymphoid tissue, dense irregular connective tissue, glandular, adipose and muscular tissues. In the superficial parts, dense irregular connective tissue extended into the core of the tonsillar papillae between the papillary pegs. Lymphoid tissue was distributed in the form numerous large and small lymphatic nodules of different dimensions along with CLU's. Some of the nodules presented darker outer corona and inner germinal centre. SEM study of lingual tonsil of pig revealed the surface of all the tonsils was covered by folds arranged in longitudinal directions. TEM study of lingual tonsils revealed the stratified squamous keratinized epithelium with different layers of stratification. Cells of stratification were attached each other by desmosomes.

Keywords: Pig, lingual tonsil, lymphatic nodules, crypts, lymphoepithelium

Introduction
A tonsil is defined as a complex organ that contained a crypt surrounded by dense lymphoid tissue and is organised in well-circumscribed aggregations of mainly B- lymphocytes, officially called lymph nodules which were separated by inter-follicular, or better inter-nodular, regions where T cells were predominant in chicken (Olah *et al*., 2003) [13].

Tonsillar follicles occurred in the bovine lingual tonsil where the openings of the bulging tonsillar follicles were macroscopically visible at the mucosal surface (Habel, 1992; World Association of Veterinary Anatomists, 2005; Casteleyn *et al*., 2007; Cocquyt *et al*., 2008; Rabmann *et al*., 2010) [7, 22, 2, 4, 19].

Cocquyt *et al*. (2005) [3] reported that the lingual tonsil were not visible in sheep.

Kumar and Timoney (2005a) [10] found that the lingual tonsil of horse located at the root of the tongue consisted of an irregular surface with rounded elevations, numerous folds and crypts.

Schaller (1992) [20], Cocquyt *et al*. (2008) [4] and Rebmann *et al*. (2010) [19] reported in bovine that the lingual tonsils were located on surface of the root of the tongue root surface and were rugged with numerous small visible openings.

Casteleyn *et al*. (2011) [1] observed that the lingual tonsils were more prominent in pigs compared to small ruminants but less developed compared to ox. It consisted of encapsulated lymphoid clusters within the mechanical papillae at the root of the tongue, caudal to the gustatory vallate and foliate papillae.

Histological examination of lingual tonsil revealed that small concentrations of lymphoid tissue on both sides of the dorsal part of tongue mainly in the connective tissue cores of the gustatory vallate papilla (Cocquyt *et al*., 2005) [3].

Girgiri and Kumar (2019a) [5] in buffalo observed that the lingual tonsil was lined by stratified squamous keratinized epithelium towards outer surface. The free surface of the epithelium was uneven, irregular and uniform, whereas the deeper surface presented the papillary pegs

Cocquyt *et al*. (2008) [4] observed in bovine lingual tonsil that the stratified squamous

epithelium of the crypts formed by a reticular epithelium heavily infiltrated by numerous lymphocytes and the tonsillar crypts were about 2000 to 2500 µm deep.

Deep crypts were found between adjacent tonsillar papillae which were lined by a stratified squamous keratinized epithelium. Lymphoid cell infiltration was observed within the crypt epithelium of the lingual tonsil of pigs (Casteleyn *et al*., 2011) [1]

Kumar and Kumar (2005) [9] reported that the lingual tonsil of goat consisted of dense lymphoid tissue and few lymphoid follicles of different dimensions. These follicles possessed a peripheral darkly stained corona and separated from each other by a dense meshwork of connective tissue consisted of reticular fibres, collagen, elastic and nerve fibres.

The bovine lingual tonsil was encapsulated but the ovine lingual tonsil was not encapsulated with connective tissue (Casteleyn *et al*., 2007) [2].

Cocquyt *et al*. (2008) [4] reported in bovine that large amount of lymphoid tissue including primary and secondary lymph nodules and diffuse accumulations of lymphocytes were present in the subepithelial connective tissue of lingual tonsils. Lymphoid tissue was in the form of organized follicles which were 5 mm in length and 3mm in diameter.

Most of the antigens that are encountered by the 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 surfaces and is composed of the mucosa associated lymphoid tissue (MALT) which represents a first line of defence. Piggery is the most efficient way of meat production, popular due to the lesser investment required on building and equipments and utilization of kitchen and hotel wastes for feeding system.

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. Pig's habit of scavenging through its snout makes most of the antigens enter the body through nasal and oral route forming a strong immune system at the mucosal sites. A detailed gross, histology and ultrastructural study on the lingual tonsils of pig would be helpful tool in mucosal vaccine developmental studies.

Materials and Methods

The present research work on grossmorphology, histomorphology and ultrastructural features of oropharyngeal and nasopharyngeal tonsils of pigs was conducted in the Department of Veterinary Anatomy and Histology, Veterinary College, Hebbal, Bengaluru, Karnataka on eight apparently healthy adult pigs of age ranging from seven to nine months. The tissue samples required for the work were collected from local slaughter house. The median sections of the heads were taken using Bone and Meat cutting machine. Both the halves were washed thoroughly in normal saline. Then the tissue samples were dissected out of the head and fixed in 10 per cent neutral buffered formalin. Tissue samples were processed as such by routine histological methods, paraffin blocks were made and sectioned to a thickness of 4-6µm. The tissues were stained with Haematoxylin and Eosin (H and E) staining technique for routine histological studies (Luna, 1968) [11], Masson's Trichrome method for demonstration of connective tissue fibres (Luna, 1968) [11], Toluidine blue and Unna's method for Mast cells (Luna, 1968) [11], Gomori's (Luna, 1968) [11] method for reticular fibres, Dane's and Ayoub Shklar method for keratin and prekeratin (Luna, 1968) [11], Methyl Green-Pyronin Stain for demonstration of plasma cells (Opstad, 1959) [14], Acid alpha naphthyl acetate (ANAE) technique for histochemical identification of T lymphocytes (Ranki *et al*., 1976) [17]. Micrometric parameters were recorded using digital camera software Prokyon Gryphax and photographs were taken by using Olympus microscope attached with a digital camera.

For Scanning electron microscopic study, tissue samples were fixed in 2.5% glutaraldehyde at room temperature for 12 hours. Later the samples were transferred to 4°C for up to 48 hours followed by 1X PBS washing twice at the interval of 30 minutes each. Then the samples were postfixed in 1% osmium tetroxide for 2 hours at 4°C which was followed by 1X PBS washing twice for 30 minutes each. Later the samples were dehydrated with graded ethanol (20%, 40%, 60%, 80% and 100%) at an interval of 30 minutes each Shapiro *et al*. (2019) [21].

Later the samples were subjected to critical point drying for up to 3 hours to reach a critical point CO2 then later CO2 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 scanning electron microscopy chamber (Zeiss 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 at Central instrumentation facility (CIF), GKVK, Bengaluru.

For transmission electron microscopy the tissue samples were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) followed by post fixation in one per cent aqueous osmium tetroxide. The specimens were dehydrated in ascending grades of ethanol, cleared in propylene oxide and embedded in araldite. Semi-thin sections of 1µ thick were cut and stained with toluidine blue for examination. Ultrathin sections 60-80 nm thick were cut and sections were harvested on carbon-coated 300-wire mesh copper grids, stained with Uranyl acetate and examined under transmission electron microscope (JEOL, Tokyo) at different magnifications at Common research facility, Electron microscope lab, NIMHANS, Bengaluru.

Results and Discussion

The lingual tonsils in pig were not visible macroscopically even after the treatment with 2% acetic acid for four hours but histologically lingual tonsils were seen within the connective tissue cores of the mechanical tonsillar papillae located caudal to the vallate papillae present at the root of the tongue (Fig. 1 and 2). These tonsillar papillae were distributed thickly at the route of the tongue in the pig similar to the observation made by Ranjit *et al.* (2015a) [16] in pigs, Casteleyn *et al.* (2011) [1] in sheep and Girgiri and Kumar (2019a) [5] in buffalo whereas Casteleyn *et al.* (2011) [1] in cattle reported that the lingual tonsil was macroscopically visible rostrolaterally orientated rows of tonsillar fossules at the root of the tongue, caudal to the vallate papillae.

Lingual tonsil of pig consisted of surface epithelium, FAE and proper tonsillar tissue.

Surface epithelium of lingual tonsil of pig was stratified squamous keratinized epithelium consisted of basement

membrane, stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. Keratinization of epithelia was only seen at the tip of the tonsillar papillae whereas no keratinization was found at the cryptic surface. The reticular fibres were observed in the basement membrane of the lining epithelium over the lingual tonsil of pig. The stratum basale was composed of a single layer of pyramidal cells with oval nuclei and dense heterochromatin and slightly basophilic cytoplasm. Cells of the stratum spinosum were arranged in 10 to 12 layers. The nucleus of the deepest cell layer was similar to those of the stratum basale. However, towards the superficial layers, cells showed round to oval nuclei and less basophilic cytoplasm. These cells were placed parallel to the longitudinal axis of the epithelium. The cells of the outermost stratum corneum were arranged in varying number of cell layers with elongated basophilic pyknotic nuclei and eosinophilic uniform cytoplasm. The lining epithelium had an uneven outer surface and the deeper cell layers had undulations which extended into the subepithelial tonsillar tissue resulting into papillary pegs in the lingual tonsil of pig (Fig. 3) which is in accordance with the observation of Girgiri and Kumar (2019a) [5] in buffalo, Kumar and Kumar (2005) [9] in goat and Ranjit *et al.* (2015a) [16] in pigs.

FAE of lingual tonsil of pig was made up of stratified squamous epithelium and extended into the crypts as crypt epithelium with some lymphoid cell infiltration. These crypts of the lingual tonsil of pigs were extending into the tonsillar tissue beneath the surface epithelium with large crypt opening with diameter of 177.87±6.75 µm and they were extended up to a length of about 1363.12±45.82 µm. Crypt epithelium was measuring about 189±4.31 µm in height. At some places the crypt epithelium was modified into follicle associated epithelium (FAE) or lymphoepithelium. In this epithelium the stratum spinosum and corneum were indistinguishable, papillary pegs were lacking and lymphoid tissue infiltration occurred and numerous lymphocytes, plasma cells, macrophages and blood capillaries were also present. In some areas of the FAE, heavy infiltration of multiple lymphoid cells makes the crypt epithelium into only one to two cells thick in the lingual tonsil of pig (Fig. 4). At the FAE areas due to heavy infiltration of lymphoid tissue, the basement membrane was interrupted and collagen fibres decreased (Fig.9) which is similar to the earlier observations of in bovine by Manesse *et al.* (1998) [12], Cocquyt *et al.* (2008) [4] and Rebmann and Gasse (2008) [19], in horse by Kumar and Timoney (2005a) [10] and in goats by Kumar and Kumar (2005) [9]. According to Perry (1994) the presence of non-epithelial cells like lymphocytes and other antigen presenting cells in the FAE of tonsils was considered a physiological characteristic in human.

Propria submucosa of the tongue of pig was characterized by dense irregular connective tissue and it was divided into two major layers. Superficial layers had lymphatic aggregations within the connective tissue and deeper layer had mucosal glands between the lingual skeletal muscle in close association with the tonsil. However, some amount of connective tissue was noticed between the lobules of the glands. Thick layer of connective tissue was noticed between the superficial and deeper layers which consisted of some amount of adipose tissue blood vessels, nerve fibres. In the superficial layer, dense irregular connective tissue extended into the core of the tonsillar papillae between the papillary pegs. Between the tonsillar follicle connective tissue was less but more at the core of the tonsillar papillae. Under the tonsillar tissue and above the glands amount of reticular and collagen fibres increased intermingled between capillaries and nerve bundles (Fig. 5).

Deeper part of tonsillar papillae consisted of lymphoid tissue, connective tissue cells, collagen fibres, nerve bundles, glandular tissue and adipose tissue. Lymphoid tissue was distributed in the form numerous large and small lymphatic nodules of different dimensions. The darker outer corona and inner germinal centre were observed in some of the nodules. Number of lymphoid nodules per microscopic field at 4x magnification were 5.00±0.46 with a diameter of 511.75±10.07 µm for large lymphoid nodules and 140.12±3.97 µm for small lymphoid nodules. Lymphoid nodules were studded with mainly lymphocytes along with some reticular cells like plasma cells, dendritic cells, macrophages. Number of lymphocytes per lymphatic nodule in the lingual tonsil of pig were about 1210.93±32.49. The lymphatic nodules were separated by parafollicular and interfollicular areas composed of a dense meshwork of reticular fibres and a few collagen and elastic fibres. The internodular space in the lingual tonsil of pig was measured about 72.00±5.11 µm. In the interfollicular areas lymphocytes, plasma cells, macrophages, blood capillaries and high endothelial venules (HEVs) were seen. Mast cells were irregularly distributed in the tonsillar tissue.

Lymphoid tissue in the lingual tonsil of pig was organized as aggregated lymphoid nodules which were encapsulated by connective tissue surrounding an epithelial crypt called cryptolymphatic units (CLU) and tonsillar nodules which are devoid of crypts were seen. A connective tissue capsule consisting of a dense arrangement of collagen and fine reticular fibres surrounded the CLU and separates them from the surrounding adipose tissue and glands. Connective tissue septa arose from the capsule and penetrated into the lymphoid tissue (Fig. 6). The mucous glandular acini were seen in the deeper parts of the propria submucosa of the tongue were in close association with the lingual tonsils.

In the present study, the surface epithelium of the lingual tonsil of pig and follicle associated epithelial cells did not show PAS activity, whereas the basement membrane showed weak PAS-positive reaction indicative of degree of presence of neutral mucopolysaccharides in these areas is similar to the findings of Ranjit *et al.* (2015a) [16] in pigs, Kumar and Kumar (2005) [9] in goat and Girgiri and Kumar (2019a) [5] in buffaloes and Kumar and Timoney (2005a) [10] in horse.

A mild reaction for ANAE in the surface epithelium and a strong ANAE rection in the parafollicular area was seen in the lingual tonsil in the present study indicating the degree of presence of T-lymphocytes in these areas which is in accordance with the earlier observations of Knowles and Holck (1978) [8] in human spleen and adenoids

Scanning Electron Microscopy (SEM)

Under SEM study, different mechanical papillae were observed at the surface epithelium consisted of lingual tonsil in pig. The surface epithelium was folded, highly irregular and projected in different directions. The longitudinal folds were separated by shallow depressions or groves. The squamous cells delineated from adjacent cells and desquamated in some areas were observed. Further, on the surface micro plicae were arranged in different patterns which

were closed type resembling human fingerprint in the present study (Fig. 7, 8 and 9) is in accordance with the earlier observations of Kumar and Kumar (2005) [9] in goats, Kumar and Timoney (2005a) [10] in horse and Girgiri and Kumar (2020a) [6] in buffaloes and Ranjit *et al.* (2015a) [16] in pigs.

Transmission Electron Microscopy (TEM)

Under TEM study, stratum spinosal cells were arranged as layers of flat elongated cells with electron dense nucleus. These cells were attached by desmosomal attachments. Cells of the stratum granulosum were electron dense and were indistinguishable from the cells of stratum lucidum and corneum in the present study (Fig.10) which is similar to the earlier observations of Girgiri and Kumar (2020a) [6] in buffaloes, Kumar and Timoney (2005a) [10] in horse.

The follicle associated epithelium having stratified squamous non-keratinized epithelium presented features of stratum basale and spinosum similar to those of the surface epithelium as reported by Girgiri and Kumar (2020a) [6] in buffalo and in horse by Kumar and Timoney (2005a) [10] as the features of strata basale and spinosum of FAE resembled those of the surface epithelium.

In the present study under TEM, the propria-submucosa of the tonsillar papillae of tongue constituted by fine HEV's lymphoid cells, fibroblasts, plasma cells, dendritic cells, macrophages neutrophils and mast cells. The plasma cell showing typical cartwheel appearance of the nuclei and abundance of Rough Endoplasmic Reticulum (RER) interspersed in between the lymphoid cells. The macrophage showing engulfed material also observed in the subepithelial portion of tongue which is associated with lingual tonsil of pig. Mast cells were observed in the parafollicular area (Fig. 11, 12 and 13). At some places fine blood capillaries and pericytes were also observed which is similar to the observations of Kumar and Timoney (2005a) [10] in horse, Girgiri and Kumar (2020a) [6] in buffaloes.

To conclude, lingual tonsils in pigs was not visible macroscopically but was located in connective tissue cores of the mechanical tonsillar papillae at the root of the tongue. Well organised lymphoid tissue in the form of small and large lymphoid nodules with large number of lymphocytes and other antigen presenting cells like macrophages, dendritic cells, mast cells make the lingual tonsil as perfect protective barrier at the oropharyngeal area to fight against the antigens entering through the oral route by mounting a strong immune response. This creates basic data for the development of oral vaccines for the various diseases than the parenteral vaccines.

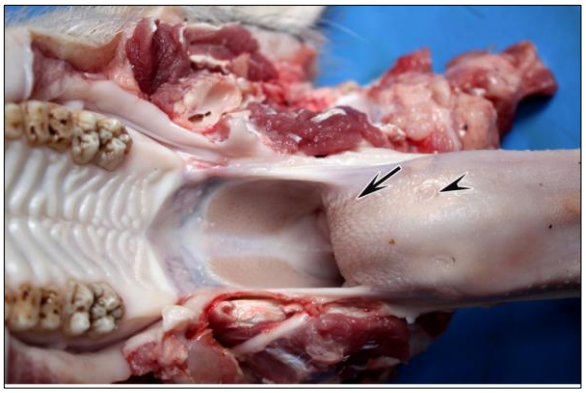

Fig 1: Gross photograph of pig head (horizontal section) showing anatomical locations of tonsillar conical papillae (arrow) representing lingual tonsils caudal to the circumvallate papillae (arrow head)

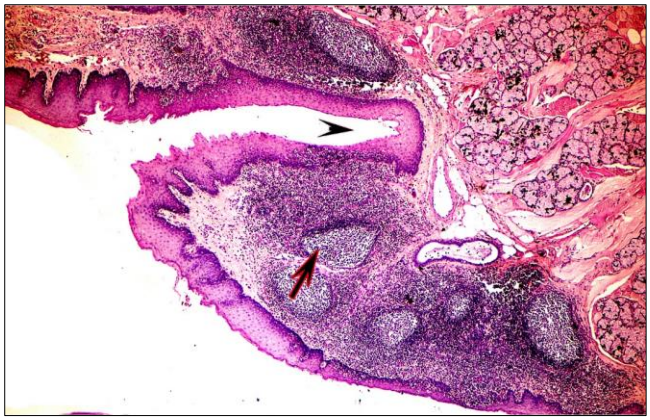

Fig 2: Photomicrograph showing the lingual tonsil of pig in the connective tissue of the tonsillar papillae (arrow)and non-keratinized epithelium in the tonsillar crypt (arrow head) H & E 4x

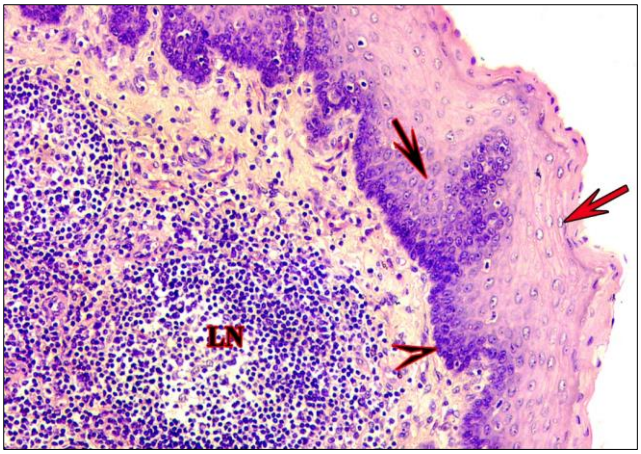

Fig 3: Photomicrograph of surface epithelium of lingual tonsil of pig showing different layers of stratified squamous keratinized epithelium. stratum basale (arrow head), stratum spinosum (arrow), stratum granulosum (red arrow) and the lymphatic nodule in the subepithelial area H & E 20x

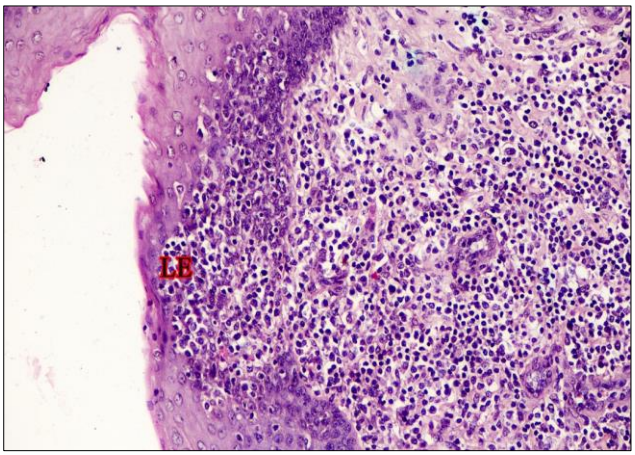

Fig 4: Photomicrograph of FAE of lingual tonsil showing lymphoepithelium (LE), lymphocyte (blue arrow), plasma cell (black arrow), macrophage (blue arrow head) and capillary (black arrow head) H & E 20x

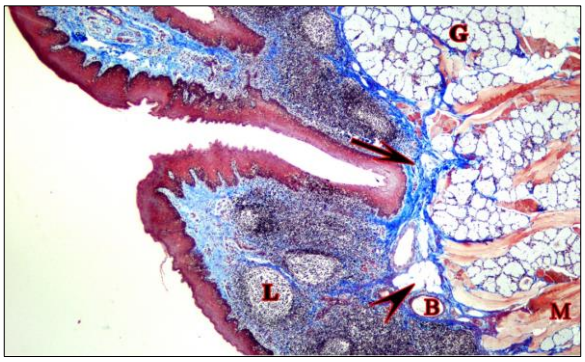

Fig 5: Photomicrograph of tonsillar papillae pig showing lymphatic nodule (L), connective tissue (arrow), blood vessel (B), glandular tissue (G), adipose tissue (arrow head) and muscle fibres (M) in lingual tonsil. Masson's Trichrome 4x

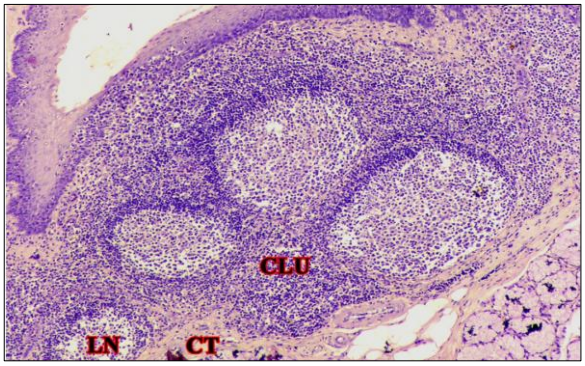

Fig 6: Photomicrograph showing cryptolymphatic units (CLU), free Lymphoid nodules (LN) separated by connective tissue capsule (CT) in the lingual tonsil of pig H & E 20x

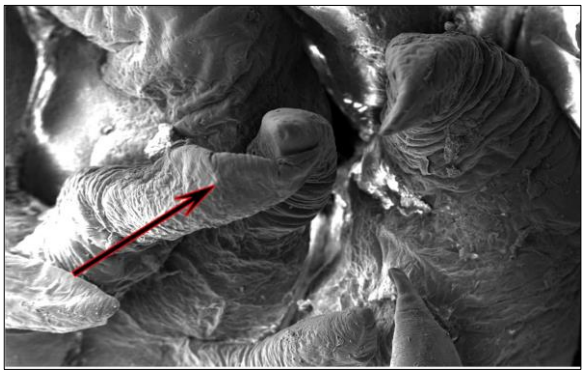

Fig 7: Scanning electron micrograph showing tonsillar papillae (arrow) consisting of lingual tonsils of pig 85x

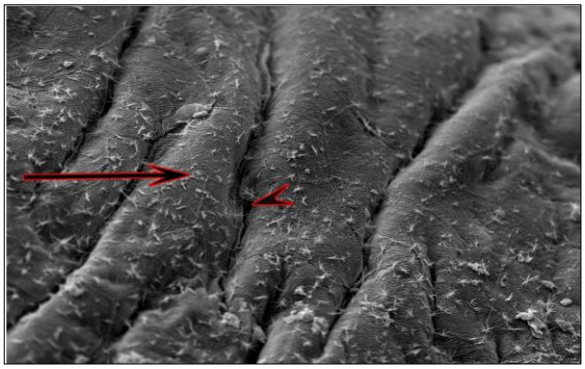

Fig 8: Scanning electron micrograph of surface epithelium showing highly irregular folds (arrow) separated by grooves (arrow head) over the lingual tonsil of pig 2600x

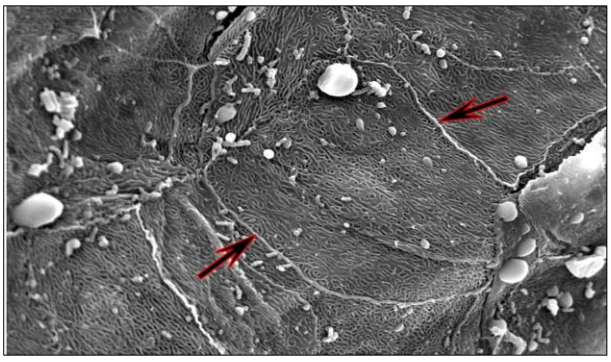

Fig 9: Scanning electron micrograph of surface epithelium showing the squamous cell arrangement well demarcated by distinct border (arrows) over the lingual tonsil of pig 4000x

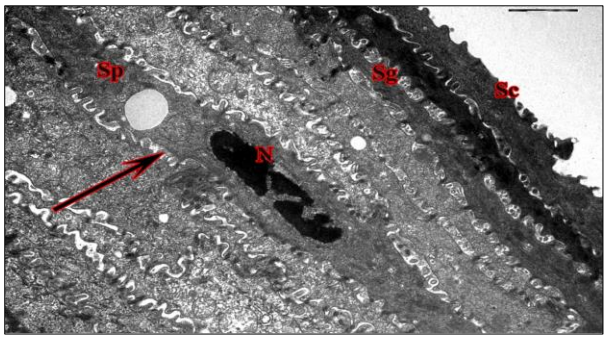

Fig 10: Transmission electron micrograph showing stratified squamous keratinized epithelium over the lingual tonsil of pig. Stratum spinosum (Sp) layer with horizontally oriented nuclei (N) with desmosomal attachment (arrow), stratum granulosum (Sg) and stratum corneum (Sc) 1500x

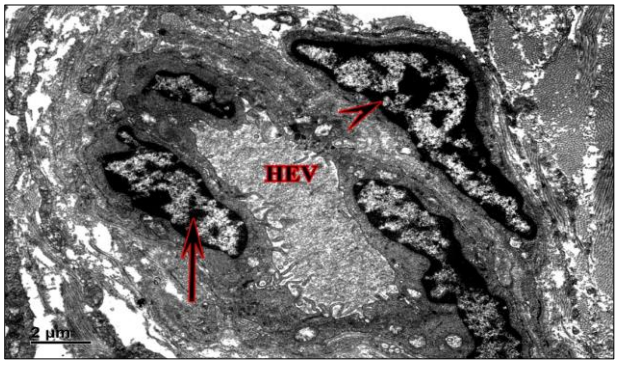

Fig 11: Transmission electron micrograph showing high endothelial venule (HEV) having endothelium (arrow) along with a pericyte (arrow head) in parafollicular area of lingual tonsil of pig. 1200x

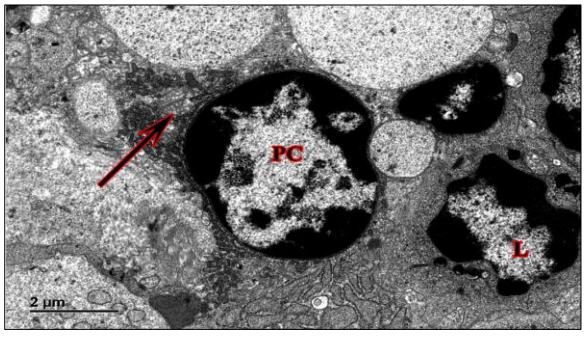

Fig 12: Transmission electron micrograph showing plasma cell (PC) with typical cartwheel appearance of nucleus and abundant RER (arrow)) adjacent to the lymphocyte (L) of the lingual tonsil of pig 2000x

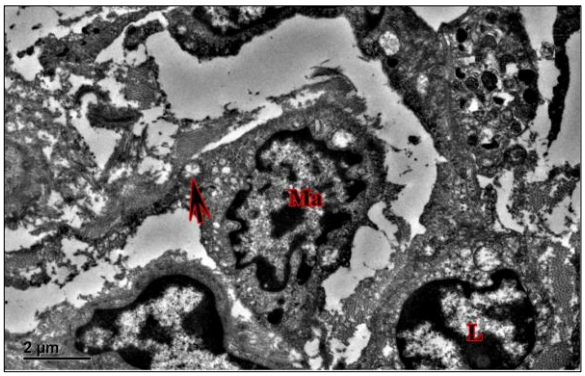

Fig 13: Transmission electron micrograph showing macrophage (arrow) with engulfed material (arrow) and an adjacent lymphocyte (L) in the lingual tonsil of pig 1500x

Acknowledgements

1. National Institute of Mental Health and Neuro Sciences, Bengaluru
2. Central instrumentation facility, GKVK, Bengaluru

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