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Well-developed immunological tissue is present in the rostral oral cavity of horses as revealed by histological and immunohistochemical examination

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ABSTRACT

Horses have a well-developed mucosal-associated lymphoid tissue in the naso-oropharynx for immunological defence and the development of immunological tolerance. The different components of this lymphoid tissue have been documented, but not all areas of the equine oral cavity have been investigated. In the present study, samples for histological and immunohistochemical examinations were collected from slaughtered horses of different ages, focusing on the rostral part of the oral cavity. Dense lymphatic tissue was found in the mucosa covering the bar area of the mandibles and the floor of the oral cavity, and it was present in horses of different ages. The most prominent lymphatic tissue, with large aggregates of lymph nodules, was present on either side of the lingual frenulum. The rostral location of this lymphatic tissue in horses renders support for application of antigens in allergen-specific immunotherapy via the oral mucosa.

Keywords: Anatomy, equine, Icelandic horses, immunotherapy, mucosal-associated lymphoid tissue, oral cavity.

YFIRLIT

Hross eru með vel þróaðan slímutengdan eitilvef í nef- og munnkoki þar sem ónæmisvörn og ónæmisþol myndast. Hinum mismunandi þáttum þessa eitilvefs hefur verið lýst en ekki öll svæði munnhols hrossa hafa verið skoðuð. Í þessari rannsókn voru sýni fyrir vefjaskoðun og mótefnalitun tekin úr sláturhrossum á mismunandi aldri, með áherslu á trjónulæga hluta munnholsins. Þéttur eitilvefur fannst í slímhúðinni sem þekur tannlausa bilið í neðri kjálka og í grennd við tunguhaftið. Eitilvefurinn var til staðar í hrossum á ólíkum aldri. Umfangsmesta eitilvefinn, eða safneitlinga, var að finna sitt hvorum megin við tunguhaftið. Staðsetning þessa eitilvefs trjónulægt í munnholinu rennir stoðum undir möguleika þess að hægt sé að þróa ónæmismeðferð um munnslímhúð hrossa.

INTRODUCTION

Mucosal surfaces of the body are endowed with lymphatic tissue for immunological defence and the development of immunological tolerance. An important part of the mucosal immune

system is the mucosal-associated lymphoid tissue (MALT) consisting of tonsils and lymph nodules (Casteleyn et al. 2011, Kumar & Timoney 2005b-d, Kumar & Timoney 2006, Liebler-Tenorio & Pabst 2006). Tonsils, an important component of MALT, are composed of B-cell rich lymphoid follicles and T-cell-dependent interfollicular areas, with close association with the mucosal surface (Liebler-Tenorio & Pabst 2006). MHC-II positive antigen-presenting cells are also an important component of MALT, of which dendritic cells (DCs) are the most efficient cell type (Reinartz et al. 2016). The presence, location, extent, and structure of MALT, including the ring of lymphoid tissue in the naso-oropharynx known as the Waldeyer's ring, varies among different domestic animal species (Casteleyn et al. 2011, Kumar & Timoney 2005b-d, Kumar & Timoney 2006, Liebler-Tenorio & Pabst 2006).

The oral mucosa plays an important role in the development of immunological tolerance. It has been suggested that MALT is biased towards tolerance to antigens due to the extensive exposure to commensal bacteria, food, and environmental material (Reinartz et al. 2016). These qualities make the oral mucosa an attractive site for allergen immunotherapy (AIT) and sublingual immunotherapy (SLIT) is being practiced in humans as an alternative to subcutaneous injections (SCIT) (Dorofeeva et al. 2021, Passalacqua et al 2020). SLIT is currently being developed to treat equine insect bite hypersensitivity (IBH), using transgenic barley and specially designed bits which prolong the time of the barley in the mouth (Jonsdottir et al. 2017). In connection with this research, a pilot study to explore the equine oral cavity was executed, in which an extensive sampling of the oral mucosa of two Icelandic horses was performed (Tryggvason L 2015). The study revealed organized lymphatic tissue present in the mucosa covering the bars of the mandibles and beneath the tongue, in addition to the known tonsillar tissues of the Waldeyer's ring (Casteleyn et al. 2011, Kumar & Timoney 2005b-d, Kumar & Timoney 2006, Liebler-Tenorio & Pabst 2006).

The aim of the present study was to investigate the presence of MALT in the sublingual and bar areas of the mandibles of Icelandic horses of different ages and the cellular components of this lymphatic tissue.

MATERIAL AND METHODS

Heads of two healthy Icelandic horses, a 4-5-year-old colt and a 5-6-month-old foal, were obtained from an abattoir and brought to the Institute at Keldur, where multiple samples from the oral mucosa were collected. Specimens for histology and immunohistochemistry (IHC) were dissected from the floor of the oral cavity beneath the free part of the tongue, including one on either side of, and two rostral to, the frenulum. Four sampling sites were at the gingival-buccal junction along the bars of the mandibles (Figure 1). Two specimens were taken from each location, with one specimen immersed in 10% neutral-buffered formalin and the other in Formalin Free Fixative, Accustain™ (Sigma-Aldrich Co, A542). Procuring Accustain fixed tissue was deemed necessary as antibodies work variably well in formalin-fixed tissue.

Additional material was collected at an abattoir, where tissue samples were dissected from the same areas of the oral cavity from 5 Icelandic slaughtered horses aged from 11 to 22 years. The samples were fixed in 10% neutral-buffered formalin for histology.

Formalin- and Accustain-fixed tissues were trimmed within 5 days after fixation, processed routinely, paraffin embedded, sectioned at 4 mm, mounted on Superfrost microscope slides and stained with haematoxylin-eosin (HE) for histological examination. Cut sections of the formalin- and Accustain-fixed samples from the colt and the foal were also collected on Starfrost slides for IHC (Table 1).

For IHC, sections of formalin- and Accustain-fixed tissue slides were incubated with the primary antibodies at dilutions 1:100 (MHC-II) for one hour at room temperature (RT), and 1:25 (CD3), 1:100 (CD20cy), and 1:25 (CD79a) overnight at 4 ˚C. The slides were then incubated for 30 minutes at RT with the relevant secondary

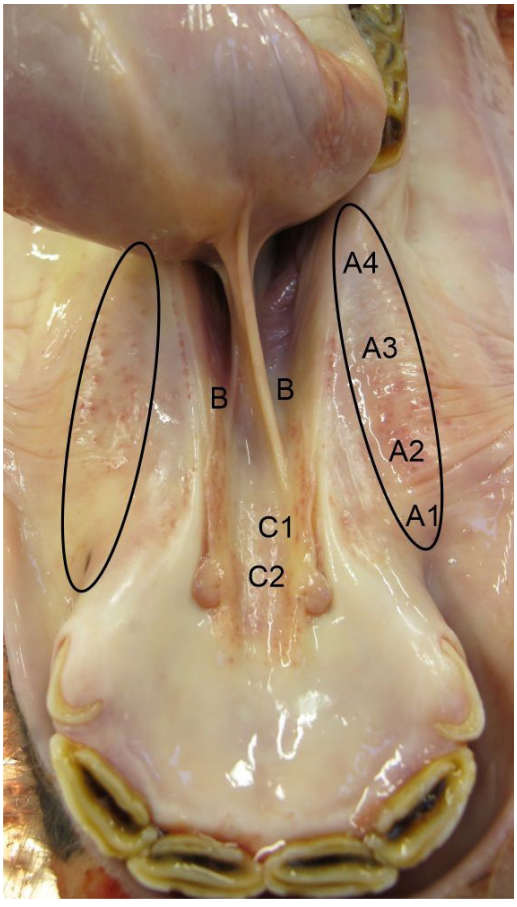

Figure 1. Oral cavity of an Icelandic foal showing areas of tissue sampling.
Four sampling sites along the buccal side of the mandibular bars (A1 – A4). Four samples from the floor of the mouth beneath the free part of the tongue: on either side of the lingual frenulum (B), and rostral to the lingual frenulum (C1 and C2).

antibodies, ready to use Kit K-1500 for MHC-II, and dilutions 1:100 (CD3) and 1:300 (CD20cy and CD79a). Streptavidin HRP (MHC-II), PAP (CD3) or Streptavidin AP (CD20cy and CD79a) was then applied, followed by the substrate solution, DAB (3,3'-diaminobenzidine) or Fast red with Levamisol. After washing, all slides were counterstained with haematoxylin. Formalin-fixed and frozen samples of equine skin, tonsil, and lymph nodes were used as positive controls. For negative controls, slides were incubated with reagent buffer in place of the primary antibody.

RESULTS

Dense lymphatic tissue was present in the lamina propria mucosa in the vicinity of the lingual frenulum and in the bar area of the mandibles of horses aged 6-months-22 years. The lymphatic tissue in the sampled areas consisted of both solitary and variably sized aggregates of lymph nodules (Figures 2a-c). There were also small clusters of loose lymphatic tissue in the superficial lamina propria mucosa, with mainly lymphocytes and the occasional plasma cell.

The lymphatic tissue associated with the tongue was present at the attachment of, and rostrally to, the frenulum (B, C1, C2 in Figure 1, Figure 2b and Figure 3c-e), with the largest aggregates of lymph nodules being on either side of the lingual frenulum (B in Figure 1, Figure 2b and Figure 3c-d). Solitary lymph nodules were the main lymphatic tissue seen in all four sampling sites of the mandibular bar

Table 1. Antibodies and immunohistochemical staining procedures.

Antibody	Clone	Secondary antibody	Detection	Substrate solution
Mouse anti-horse-MHC II[†]	CVS20	Kit K-1500[*] Rabbit & mouse	Streptavidin HRP	DAB[*]
Rabbit anti-human CD3[*]	Polyclonal	Swine Anti-rabbit[*]	Rabbit PAP[¶]	DAB
Mouse anti-human CD20cy[*]	L26	Biotin. rabbit Anti-mouse[*]	Streptavidin AP[§]	Fast Red + Levamisol[¶]
Mouse anti-human CD79a[†]	HM57	Biotin. rabbit Anti-mouse[*]	Streptavidin AP	Fast Red + Levamisol

[†] Bio-Rad Laboratories, Inc Ltd, UK
[*] Dako, Denmark
[¶] Sigma-Aldrich Chemie GmbH, Germany
[§] GE Healthcare UK Limited, UK

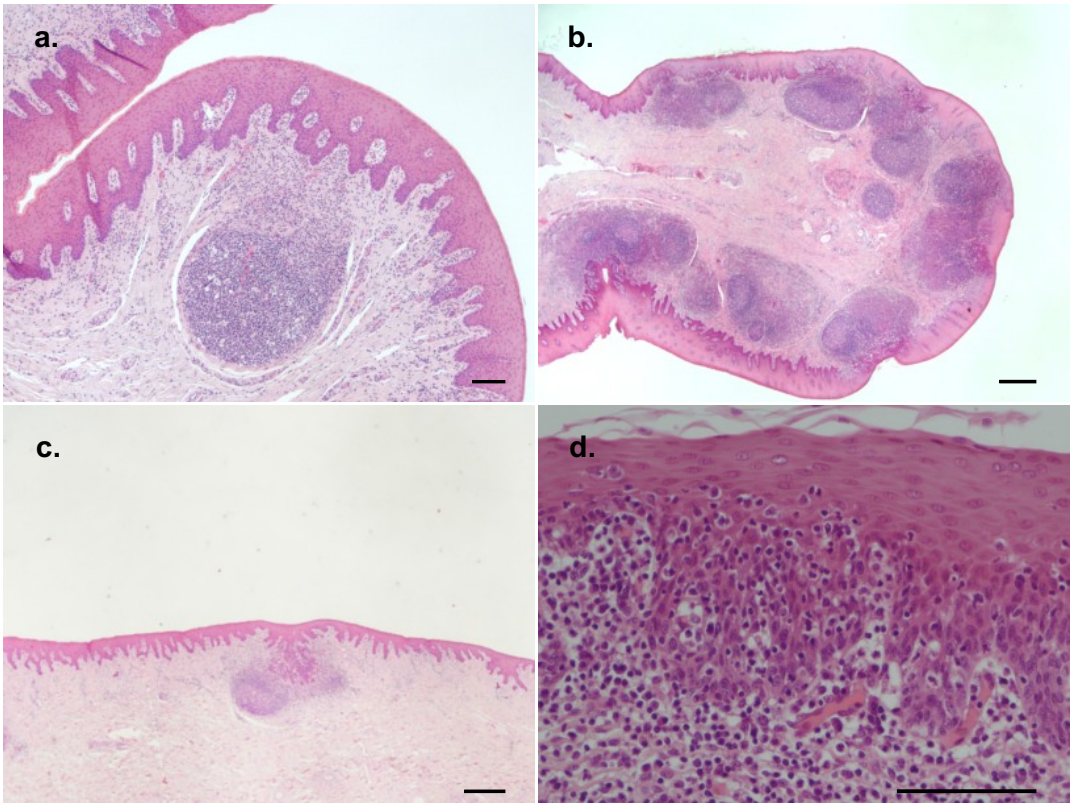

Figure 2. Representative illustration of haematoxylin-eosin-stained sections of formalin-fixed tissue sampled from the oral cavity of Icelandic horses.

a. Solitary lymph nodule in the lamina propria mucosa of the mandibular bar area; 4-5-year-old colt.
b. Aggregates of lymph nodules in the lamina propria mucosa, lateral to the lingual frenulum; 18-year-old horse.
c. Solitary lymph nodule in the lamina propria mucosa of the mandibular bar area. Irregular pegs from the mucosal epithelial lining extend down to the lymphatic tissue; 21-year-old horse.
d. The mucosal epithelial lining lateral to the lingual frenulum has irregular pegs with indistinct borders due to numerous infiltrating leukocytes; 5-6-month-old foal.

Bar = 100 mm

area (A1-4 in Figure 1, Figure 2a and c, and Figure 3a and b).

The oral cavity in these regions was lined by a non-keratinized squamous epithelium with short, plump, somewhat irregular pegs extending into the lamina propria mucosa. In areas, the epithelium was slightly indented, sometimes attenuated, and indistinct because of infiltrating leukocytes (Figures 2d and 3f), but no follicular crypts or M-cells were seen.

For the antibodies detecting MHC-II, CD3, CD20cy and CD79a, the results of IHC for formalin- and Accustain fixed samples were comparable. CD20cy and CD79a positive B-cells were the predominant cell type in the lymph nodules, with CD3 positive T-cells at the outer borders (Figures 3a-d). A mixture of B- and T-lymphocytes were in the internodular areas of aggregated lymph nodules and in the adjacent diffuse lymphatic tissue (Figure 3a-d). Many of the cells in the dense and adjacent diffuse lymphatic tissue expressed MHC-II, as well as cells around blood vessels just beneath the mucosal epithelial lining (Figure 3e). Cells

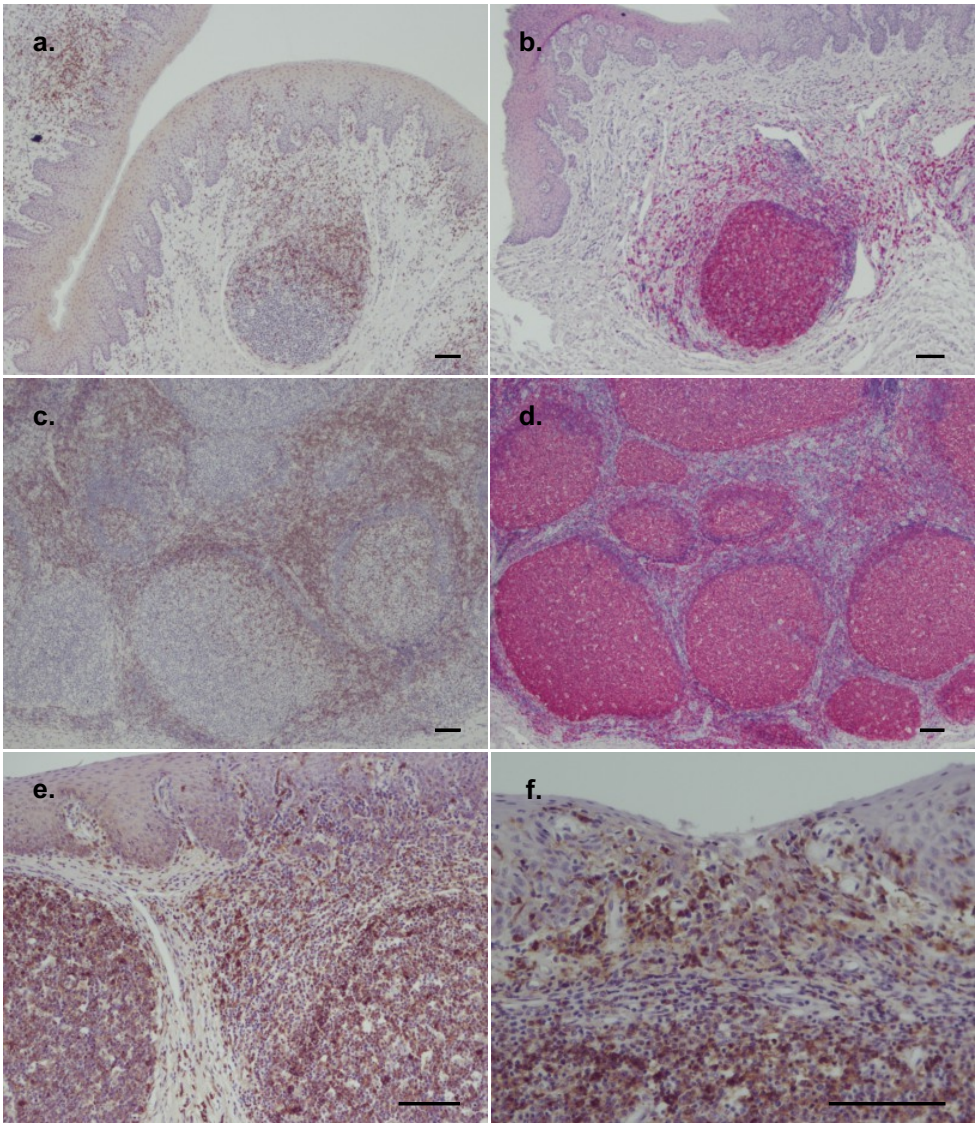

Figure 3. Representative illustration of immunohistochemical stained sections of fixed tissue sampled from the oral cavity of Icelandic horses.

a. CD3 positive T-lymphocyte form a cap towards the oral cavity on a solitary lymph nodule in the mandibular bar area, same location as in figure 2a; 4-5-year-old colt.
b. CD20yc positive B-cells are the predominant cell type in a solitary lymph nodule in the mandibular bar area and B-cells are also present in the loose lymphatic tissue surrounding the nodule; 5-6-month-old foal.
c. Aggreagates of lymph nodules lateral to the lingual frenulum. CD3 positive T- lymphocytes are at the outer borders and between the lymph nodules; 5-6-month-old foal.
d. Same area as in figure 3c with CD20yc positive B-cells prevailing in the lymph nodules.
e. Numerous MHC-II positive leukocytes in two lymph nodules and in the loose lymphatic tissue beneath the mucosal epithelium, lateral to the lingual frenulum; 4-5-year-old colt.
f. Attenuated, irregular mucosal epithelial lining in the mandibular bar area, with blurred outlines due to infiltrating MHC-II positive leukocytes; 5-6-month-old foal.

Formalin-fixed samples; figures a-d. Accustain-fixed samples; figures e and f. Bar = 100 mm

infiltrating the mucosal epithelium were MHC-II (Figure 3f) and CD3 positive, with fewer CD20cy and CD79a positive cells.

DISCUSSION

The present study verified the presence of dense lymphatic tissue in the oral mucosa beneath the tongue and in the bar area of the mandibles in Icelandic horses. Histological and immunohistochemical composition of this lymphatic tissue fulfils the criteria of MALT in general and non-cryptic tonsils in particular, corresponding to tonsillar tissue in other parts of the oral cavity (Liebler-Tenorio & Pabst 2006, Casteleyn et al 2011, Kumar & Timoney 2005b). Lymphatic tissue at these two locations of the rostral oral cavity persists into adult life and does not involute like the ileal Peyer's patches (IPP), as it was present in horses age under 1 year and up to 22 years. This study did not, however, ascertain whether this lymphatic tissue was constitutively present or whether, like the bronchial associated lymphoid tissue (BALT) in horses, it develops after antigen encounter (Liebler-Tenorio & Pabst R 2006). The mucosal lining, with its infiltrating leukocytes, also parallels the description of the follicular associated epithelium (FAE) of other tonsillar tissues (Liebler-Tenorio & Pabst 2006, Kumar & Timoney 2005a).

MHC-II positive cells were present in this lymphatic tissue and in the mucosal epithelium, but the present study could not verify whether they represented professional antigen-presenting cells. Several specific antibodies for antigen-presenting cells were tested on formalin and Accustain-fixed tissue, in addition to frozen tissue sampled from the foal and the colt, without positive results.

This paper describes MALT in the rostral oral cavity of Icelandic horses that should make allergen-specific immunotherapy via the oral mucosa feasible (Jonsdottir S 2017).

REFERENCES

Casteleyn C, Breugelmans S, Simoens P & Van den Broeck W 2011. The tonsils revisited: Review of the anatomical localization and histological characteristics of the tonsils of domestic and laboratory animals. *Clin Dev Immunol*. https://doi.10.1155/2011/472460.

Dorofeeva Y, Shilovskiy I, Tulaeva I, Focke-Tejkl M, Flicker S, Kudlay D, Khaitov M, Karsonova A, Riabova K, Karaulov A, Khanferyan R, Pickl W-F, Wekerle T & Valenta R 2021. Past, present, and future of allergen immunotherapy vaccines. *Allergy* 76, 131-149. https://doi.10.1111/all.14300.

Jonsdottir S, Svansson V, Stefansdottir S-B, Mäntylä E, Marti E & Torsteinsdottir S 2017. Oral administration of transgenic barley expressing a Culicoides allergen induces specific antibody response. *Equine Vet J* 49, 512-518. https://doi.10.1111/evj.12655.

Kumar P & Timoney J-F 2005a. Histology and ultrastructure of the equine lingual tonsil. I. Crypt epithelium and associated structures. *Anat Histol Embryol* 34, 27-33. https://doi.10.1111/j.1439-0264.2004.00560.x.

Kumar P & Timoney J-F 2005b. Histology and ultrastructure of the equine lingual tonsil. II. Lymphoid tissue and associated high endothelial venules. *Anat Histol Embryol* 34, 98-104. https://doi.10.1111/j.1439-0264.2004.00579.x.

Kumar P & Timoney J-F 2005c. Histology, immunohistochemistry and ultrastructure of the equine tubal tonsil. *Anat Histol Embryol* 34,141-148. https://doi.10.1111/j.1439-0264.2005.00582.x.

Kumar P & Timoney J-F 2005d. Histology, immunohistochemistry and ultrastructure of the equine palatine tonsil. *Anat Histol Embryol* 34, 192-198. https://doi.10.1111/j.1439-0264.2005.00594.x.

Kumar P & Timoney J-F 2006. Histology, immunohistochemistry and ultrastructure of the tonsil of the soft palate of the horse. *Anat Histol Embryol* 35, 1-6. https://doi.10.1111/j.1439-0264.2005.00622.x.

Liebler-Tenorio E-M & Pabst R 2006. MALT structure and function in farm animals. *Vet Res* 37, 257-280. https://doi.10.1051/vetres:2006001.

Passalacqua G, Bagnasco D, Canonica G-W 2020. 30 years of sublingual immunotherapy. *Allergy* 75, 1107-1120. https://doi.10.1111/all.14113.

Reinarz S-M, van Tongeren J, van Egmond D, de Groot E-J-J, Fokkens W-J & van Drunen C-M 2016. Dendritic cell subsets in oral mucosa of allergic and healthy subjects. *Plos One* 11. https://doi.10.1371/journal.one.0154409.

Tryggvason L 2015. Distribution and Characteristic of Lymphoid Tissue within the Equine oral mucosa. BVetMed Thesis (EMS No:S364). Royal Veterinary College, University of London, UK

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