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The utility of histopathology in identifying structural differences among layers of the lamina propria☆

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ABSTRACT

Objective: The purpose of this study is to assess the modality of laryngeal histopathology in identifying 1, 2, or 3 layers in the lamina propria.
Methods: Blinded analysis was performed, with a set of histophathologic slides where the magnification and localized regions shown were all standardized. Two senior pathologists with experience reviewing laryngologic histophathology were asked to assess whether the vocal fold lamina propria they evaluated contained 1, 2, or 3 layers. Their ability to accurately assess this was calculated.
Results: The first pathologist correctly identified 13 of 25 (52%) specimens. The second identified 19 of 25 (76%) specimens after receiving gold referents before the test. No significant difference was seen between the pathologists' interpretations (McNemar test; $p = 0.0833$).
Conclusion: Our results show the difficulty of using histopathology to distinguish layers in the lamina propria even when the reviewers are senior pathologists. These findings imply that more objective modalities for such analyses may be beneficial.

1. Introduction

Speech exists as a sound source emanating from our lungs through exhalation, with tonal and pitch modulation occurring at the level of our vocal folds, and the final shaping at the level of our tongue, lips, mouth, and palate. Our vocal cords, the central structure involved in vocalization, have the ability to sustain pliability during pitch variability, which may allow humans to convey subtle emotional aspects through speech. This utilization and function greatly develops as we mature. Oral communication begins in newborn infants in the form of crying and evolves to the complex language we use as adults.

The human vocal fold, differentiated from the often used and misleading term vocal cord, is a complex, multi-layered structure consisting of epithelial, lamina propria, and deeper muscular layers. In the adult human vocal fold, Hirano et al. described a three-layered lamina propria structure consisting of the superficial, middle, and deep layers (SLP, MLP, and DLP) [1]. With this description in mind, he further defined the vocal fold as having a cover (epithelium and superficial lamina propria), ligament (MLP and DLP), and body (vocalis muscle) [2]. He and others have characterized the physical properties of the lamina propria, noting the superficial layer, directly beneath the epithelium, as 'loose and pliant' lacking structural fibers, with the intermediate layer mainly consisting of elastin and the deep layer containing collagenous fibers [1–4]. As the vocalis muscle pulls the lamina propria taut, the middle and deep layers provide the structural support needed for this tension. While these two layers are stretched, the superficial lamina propria and epithelium remains flexible, allowing for the mucosal wave and vibration.

The vocal fold has different tasks as it develops from a single to three-layered lamina propria. When, why, and how this multi-layered structure develops remain unanswered questions. As a single layered structure in babies, its only function is to wail. However, as adults with a tri-layer lamina propria, we use pitch subtleties to convey nuanced messages in our speech. Both Hirano and later Hartnick noted that these structures are not present in the fetal or newborn vocal fold [2,5]. Examining younger

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specimens, Hirano reported that a human fetal lamina propria consisted of a relatively uniform layer of tissue that eventually develops into the trilaminar structure seen in the adult vocal fold. Examining larynxes of children 1–4 years old, he observed a bilaminar organization with an SLP, yet found no significant distinction between the elastin and collagen fiber layers that is seen in the mature vocal fold [5].

Although the exact timing of when the one lamina propria of the fetus matures to a two and finally three-layered structure remains unclear, work by Hartnick et al. among others suggests that a mature vocal fold is not seen until teenage years, and that a differentiation between the MLP and DLP occurs at approximately seven years of age [2,6]. It has also been shown that this development from infancy to adulthood is not a simple process. At the time of birth, there is a remarkable amount of cellular activity that leads to hyper and hypocellular regions, and a significant amount of change at the beginning stages of development [2].

There are many issues regarding the ability to draw inferences about developmental timing from histologic samples. Among these limitations include sub-epithelial fibrosis, a condition seen in many specimens commonly occurring at the level of the SLP. Additionally, the elastin and fibrous layers that differentiate the MLP and DLP are not well characterized. These factors make qualitative assessments of human vocal fold development difficult, and call into question the validity and reliability of the use of this form of data to draw conclusions. The goal of this paper is to highlight the concerns about this dilemma and assess the modality of laryngeal histopathology in identifying 1, 2, or 3 layers in the lamina propria.

2. Methods

A histologic database consisting of adult human, porcine, and human fetal vocal fold images was reviewed. All vocal folds used in the database were collected with Institutional review board approval. Specimens were preserved in 10% formalin for 24 h before being processed for histology. The vocal folds were sectioned vertically, perpendicular to the length of the tissue. A trichrome stain was used on the tissue due to its ability to distinguish cellular from extracellular components, and to allow for the characterization both of elastin as well as collagen. However, one adult human specimen with a hematoxylin–eosin stain was used when a trichrome stained slide was not available. These two stains were chosen, as they were the primary stains used in prior studies on this topic [2].

Twenty-five specimens were selected as the best examples of 1, 2, and 3 layers in the lamina propria. These included 10 human fetal, 10 porcine, and 5 adult human, and were chosen by the principal investigator and first author, who are both experienced in assessing vocal fold histology. There has been no previous literature in this area to help calculate an appropriate sample size. Therefore, in the absence of a calculated sample size we chose the 5 best specimens in each category, from a database of 12 human fetal, 88 porcine, and 74 adult human vocal folds. After selecting our best samples, we found an additional adult human and porcine specimen to include in the study. The majority of adult human specimens were excluded due to factors such as inflammation, trauma, and damage. These characteristics were seen less in the human fetal and porcine samples, and the authors decided to choose an equivalent amount to the adult human specimens. Porcine specimens were used since it has been previously published by Garrett et al. that their lamina propria is composed of two layers [7]. Work by Hirano and Hartnick have already shown substantial evidence that lamina propria of human fetal and adult vocal folds contain 1 and 3 layers respectively [2,5]. Of the 17 specimens used, there were 5 human fetal, 6 porcine, and 6 human adult specimens.

All histolopathologic images had a standardized 100× magnification and localized region of interest. They were obtained using an Olympus CX41 System Microscope with an Olympus Q Color 5 digital camera. The images were then organized into a test, where they were de-identified and randomized. Each image was placed on a separate page so they could be viewed one at a time to prevent comparisons among specimens. Examples of human fetal, porcine, and adult human images included in the test can be seen in Figs. 1–3, respectively.

Two senior pathologists with experience evaluating laryngologic histopathology were separately asked to assess the images and determine if they showed a lamina propria structure consisting of 1, 2, or 3 layers. Before reviewing the histopathology, both pathologists were given a primer on how the tissue for the slides was sectioned and what the localized region in each image showed. They were also informed the number of fetal human, porcine, and adult human samples used in the test. One top specimen from each sample type was selected and used as a gold standard reference of the lamina propria layers. These were reviewed by both pathologists before evaluating the images. The pathologists' assessment of each slide was recorded. The data were collected and analyzed using a McNemar test to compare proportions of correct sample identification between both pathologists. SAS version 9.3 (Cary, NC) was the statistical software used in this analysis.

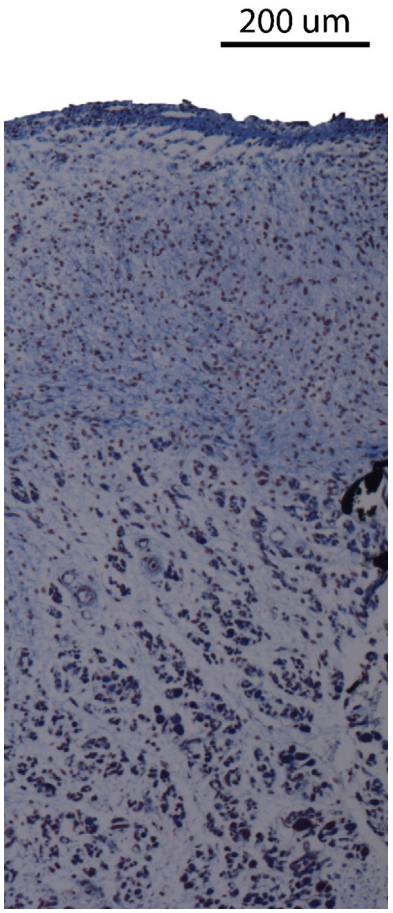

Fig. 1. Example of a human fetal (monolayer) histopathologic image both pathologists evaluated.