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Young's Modulus of Canine Vocal Fold Cover Layers

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Abstract

Objectives—The objective of this study was to measure the elastic modulus (Young's modulus) of canine vocal fold cover layers.

Study Design—Basic science study.

Methods—Cover layers from vocal folds of eight canine larynges were dissected. Cover layer samples from the mid-membranous, medial vocal fold surface area were used to measure material stiffness using a previously validated indentation method. Cover layers from two human larynges were also measured as control references. Superior and inferior medial cover layers were measured separately. A total of 15 superior medial surface and 17 inferior medial surface specimens from the canine, and 2 and 4 specimens respectively from the human, were tested.

Results—In the canine larynges, the mean Young's modulus of the superior medial surface was 4.2 kPa (range 3.0 kPa – 5.4 kPa, SD 0.6 kPa), and of the inferior medial surface was 6.8 kPa (range 5.4 – 8.5 kPa, SD 0.8 kPa). Measurements on human cover samples were 5.0 kPa (range 4.7 – 5.4 kPa, SD 0.5 kPa) and 7.0 kPa (range 6.7 – 7.3 kPa, SD 0.3 kPa) for the superior medial and inferior medial surface, respectively. Human measurements were similar to the previously validated measurements. There was no difference between the stiffness measurements in the human and canine cover layer samples ($p>0.05$).

Conclusions—The elastic stiffness (Young's modulus) of the canine and human vocal fold cover layers is similar. Findings support the use of canine larynx as an externally valid model to study voice production.

Keywords

Vocal Fold; Laryngeal physiology; Young's Modulus; Indentation

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INTRODUCTION

In vivo animal models have been used extensively to understand human laryngeal physiology.[1–2] One of the main advantages of animal models is the ability to perform invasive laryngeal experiments which would not be ethically possible in humans.[3] The canine is the most commonly used *in vivo* model in laryngeal physiology research. The advantages of the canine larynx include excellent anatomic size match of laryngeal muscles and cartilaginous framework, as well as a nearly identical neuromuscular anatomy that is experimentally accessible for neuromuscular stimulation and measurement of phonatory parameters.[4] However, despite these similarities the external validity of this model is frequently questioned as there are some differences between the larynges, such as a longer cartilaginous glottis in the canine larynx.[5] Thus, to extend the applicability of research findings from the canine model (or any other model) to human laryngeal physiology it is important to ensure that the model larynx is as similar to the human larynx as possible.

Many previous reports have compared the gross morphology of the canine larynx and other mammalian species to the human larynx. Cox et al. quantified the geometric structure of the cricothyroid (CT) and thyroarytenoid (TA) muscles in human and canine larynges.[6] They found that the basic gross function of the CT and TA muscles were the same, and although the canine CT muscle was slightly larger in cross section, the ratios of mass and cross-sectional areas of the CT and TA muscles between the two species were not significantly different. In considering this slight difference in muscle cross section it should be remembered that in these studies post-mortem human larynges come from the elderly cohort (who have higher propensity for age related vocal fold atrophy), while the canine larynx is harvested from young healthy animals. Jiang et al. compared laryngeal anatomy and function in pig, dog, deer, and human larynges.[7] They found that while vocal fold length was similar in all the animals, the best anatomic match was between the human and the canine larynx while the deer and pig were slightly longer. However, unlike the Cox study, they found the CT muscles similar in all the animals. Kim et al. compared human, canine, and sheep laryngeal dimensions and found near perfect match between the human and canine larynges in terms of overall dimensions and arc of rotation of CT and cricoarytenoid (CA) joints.[8] The ovine larynx was significantly different.

The acoustic output and phonatory aerodynamics of the larynx are dependent not only on the gross vocal fold neuromuscular anatomy, which sets up the glottic phonatory posture,[9] but also upon the specialized histopathology of the vocal fold cover layer, which facilitates self-sustained oscillation of the glottis.[10–11] The body-cover theory of phonation, which is the most contemporary paradigm for our understanding of voice production, states that the layered histology of the vocal fold can be divided biomechanically into the “body” layer, consisting of the TA muscle and the adjacent deep collagen fibers, and the “cover” layer consisting of the superficial and intermediate lamina propria layer and the vocal fold epithelium.[12] In this model the fundamental frequency of voice is primarily dependent on cover layer tension, which is controlled by muscular forces from the TA and CT muscles, if the amplitude of vocal fold motion is restricted to the cover. The cover layer elastic properties determine its response to the muscular forces affecting its tension, and small variations in elastic properties of the cover layer can result in significantly altered

phonation.[13] Thus, it is important that an ideal animal laryngeal model not only share similar overall muscular dimensions but also similar cover layer elastic properties.

Whereas there have been multiple reports comparing the gross morphology of human and canine laryngeal muscles (the "body" layer) and cartilaginous laryngeal framework, there is a relative paucity comparing the cover layer elastic properties between these species. The purpose of this study was to measure the elasticity of the canine vocal fold cover layer. Herein, we measure the Young's (elastic) modulus of the canine vocal fold cover layers using a previously validated indentation technique that was used to measure the modulus of human vocal fold cover layers.[14]

MATERIALS AND METHODS

The University of California, Los Angeles, Animal Research Committee and the Institutional Review Board approved the use of larynges for research. Eight canine larynges were obtained from humanely euthanized canines used for other approved research protocols. Each canine was of the mongrel breed between one and two years old and weighed about 20 to 25 Kg each. We also re-measured the Young's modulus of several human vocal fold cover layers to serve as internal control reference specimens. Two human larynges were harvested from autopsy cases within 48 hours post-mortem. All larynges were kept quick frozen after harvest in −80 °C freezer until the day before the experiments, when they were thawed overnight in a refrigerator at 4 °C.

On experimental day the fully thawed larynges were removed from the refrigerator and allowed to come to room temperature within closed plastic bags on a laboratory work bench for 2–3 hours. The larynges were bisected at the anterior and posterior commissures, exposing the vocal folds. Cover layers were then sharply dissected off from both vocal folds of each larynx using fine iris scissors and 3.5X magnification from surgical loupes. The cover layer excision proceeded from the infraglottic vocal fold towards the superior medial vocal fold margin, then laterally towards the ventricle on the superior surface of the vocal fold. During the dissection care was given not to take any fibers of the TA muscle, which was left attached to the larynx.

The mid-membranous areas of the dissected cover layers were then separated into their three vocal fold surface parts: (1) superior surface, (2) superior medial surface (superior 3–4 mm of the medial vocal fold surface cover layer), and (3) inferior medial surface (inferior 3–4 mm of the medial vocal fold surface cover layer). Measurements of Young's modulus were made immediately after dissection. A detailed theoretical background and methodological considerations of the indentation technique for measurement of vocal fold elastic modulus was described previously and the same apparatus and methodology were used in this study.[14] Samples were placed on the indenter platform with the epithelium surface facing up towards the indenter, and kept in contact but not submerged in 0.9% saline solution to keep moist and prevent desiccation (Figure 1). The indenter was mounted onto a force transducer (Shimpo DF-0.5R, 220 grams load cell, Shimpo Instruments, Itasca, IL), which was mounted onto a motorized linear traverse (Model MA2506W1- S2.5-0, Velmex, Bloomfield, NY). The motorized linear traverse moved the cylindrical indenter into the sample in a

direction perpendicular to the sample. Measurements were made with the 1 mm diameter indenter from the middle of each specimen. The voltage from the strain gauge of the force transducer was amplified by a factor of 100 and recorded with a PC-based AD board (UEI PowerDAQ, 16 bit resolution of 10 Volt input span, 2000 Hz sampling rate). Before beginning each measurement the indenter was manually positioned as close as possible to the testing sample, without making contact. During measurements, the indenter was moved by the traverse in steps of 0.02 mm towards the testing sample (loading) and then moved back to its original position (unloading). After a wait time of 1.5 second after each traverse movement, the average of the force signal over 0.5 sec was recorded as the indentation force (*F)* for the imposed indentation depth (*h)*.

To maintain consistency of the procedural and measurement techniques with previous indentation measurements on the human larynx, human vocal fold covers served as positive controls. Young's moduli of human vocal fold covers were measured first to ensure that measurements were consistent with previously published data.[14] Once this was confirmed, measurements of the canine samples immediately followed. For each cover sample, Young's modulus was calculated from the slope of the initial portion of the unloading indentation cycle (dF/dh) based on the Hertzian model for a cylindrical contact as described previously.[14] The optimal indentation depth for measurements was about 0.08 mm. This depth was determined after performing numerous loading-unloading cycles and was the indentation depth with linear dF/dh relationship and optimal signal to noise ratio. An indentation depth close to 10% of the total sample thickness has been consistently found to be optimal for measuring Young's modulus using the indentation technique.[14] For each sample, indentation measurements were performed for five cycles and the data averaged. Standard deviation was calculated for each group and analyzed using ANOVA statistical analysis. Additionally, the results were compared using two-tailed t-tests with concurrently obtained human data as well as our previously published data on human vocal fold cover stiffness measurements using the same indentation technique and apparatus.[14]

RESULTS

The superior surface of the canine vocal fold cover was measured to be too thin (<1mm) to be used to accurately measure Young's modulus using the indentation technique, as the sample thickness needs to be at least 10 times the indentation depth to obtain accurate measurements, as discussed and explained previously. [14] Thus, measurements were not performed on the superior surface cover layer. The thickness of the medial vocal fold surface cover layers were all between 1.5 and 2 mm and were suitable for measurements. The mid-membranous medial surface cover layer of each vocal fold was then divided into superior medial and inferior medial halves. Each measured cover sample was approximately 4 mm × 4 mm.

15 superior medial and 17 inferior medial mid-membranous cover layer samples were adequately prepared from the eight canine larynges. In one vocal fold the inferior medial cover was not suitable for measurements while in another vocal fold two good samples of the inferior medial cover layers were obtained. Mean calculated superior medial cover layer Young's Modulus was 4.2 kPa (range 3.0 kPa – 5.4 kPa, SD 0.6 kPa) and mean inferior

medial cover layer Young's Modulus was 6.8 kPa (range 5.6 – 8.5 kPa, SD 0.8 kPa) (Figure 2). Measurements of human cover samples averaged 5.0 kPa for superior medial surface (Range 4.7 – 5.4 kPa, SD 0.5 kPa) and 7.0 kPa (range 6.7 – 7.3 kPa, SD 0.3 kPa) for the inferior medial surface. All measurements are listed in Table 1. There was no statistical difference between Young's modulus calculated from the human versus canine cover samples for the respective areas ($p > 0.05$).

DISCUSSION

The acoustic and aerodynamic properties of voice such as amplitude, frequency, vocal range, subglottal pressure, and airflow requirements are entirely dependent on the anatomic dimensions (length and width of the vocal folds, range of motion of laryngeal joints, etc.) and material properties (e.g. Young's modulus) of the vibratory tissue. As it is ethically unacceptable to perform invasive neuromuscular stimulation on humans, animal models remain essential for these studies. The external validity of the canine larynx and other in vivo models has been debated ever since non-human models were used to understand human laryngeal physiology. The functional control aspect of voice production is dependent on the neuromuscular anatomy, i.e. do the canine larynx and the human larynx share the same neuromuscular anatomy? In regards to the neuromuscular anatomy, numerous studies have shown that the canine larynx is nearly identical to the human larynx as it is a close match in terms of its gross, microscopic, and histologic anatomy, with the main difference being that the interarytenoid muscle is less developed and the cartilaginous glottis is more prominent. [4, 13–17] However, the canine vocal fold cover layer material properties, which would have a significant impact on mucosal wave characteristics and acoustic output, have not been measured using a validated technique.

This study found nearly identical Young's moduli between the canine and human larynx cover layers and thus supports the notion that the canine in vivo model is a valid model for laryngeal physiology research. Human and canine vocal folds have nearly identical Young's moduli and they both retain the differential stiffness between the superior and inferior medial surface (Figure 2). In retrospect this finding may not be surprising as there is ample indirect evidence that material properties of human and canine should be similar. Mucosal wave properties of the larynx was compared by Regner et al. between ex vivo bovine, canine, ovine, and porcine laryngeal vibration and in vivo human laryngeal vibration using high speed digital imaging.[18] They reported that "no statistically significant differences were found with respect to frequency, amplitude, or phase difference between canines and humans. Porcines were not significantly different from human females but did have an oscillation frequency significantly different from human males. Ovine vibrational amplitudes were significantly different from humans, and bovine frequency and amplitude differed significantly from humans." Thus, canine larynges were "the most appropriate specimens for laryngeal studies contingent on vibratory or kinetic properties of phonation".

While we found similar cover layer stiffness between the canine and human larynx, the literature is not consistent regarding the microanatomy of canine and human cover layers. For example, while Kurita et al found a 3 mm thick, double-layered lamina propria (LP) layer with a poorly developed vocal ligament in the canine larynx, Garrett et al found a

trilaminar LP layer that resembled the human larynx.[19–20] In particular, Garrett et al found similar intermediate layers in both human and canine species (dense ground substance over dense elastin), while the deep LP layer differed only in that the canine had more ground substance over collagen as compared to the human larynx, which had mostly collagen.[20] The thickness of the cover layers between canine and human were also more similar in the Garrett study. Perhaps the canine species used accounts for this difference. In regards to the functional role of the less developed vocal ligament it has been suggested that better developed vocal ligament (intermediate and deep layers of the lamina propria layer) of the human larynx hypothetically allows the larynx to handle more longitudinal stress, thus facilitating the production of higher fundamental frequencies in the upper vocal registers of the singing voice.[21] In our previous investigations on F0 control using the in vivo canine model we have found fundamental frequencies ranging from 80 Hz to776 Hz, which covers the human speaking and singing range very well.[3, 22] Therefore, the material properties of the cover layer and the neuromuscular anatomy of the canine larynx makes it a suitable externally valid model to study laryngeal physiology.

There are some limitations to using the results from the indentation technique used in this study in regards to phonatory modelling. Ideally, we want to know the material stiffness of the vocal fold at its typical phonatory frequency, e.g. 100–200 Hz. The indentation technique we used utilized an indenter to compress tissue perpendicularly at 0 Hz. Thus results are applicable for zero frequency of vibration. Technical and methodological improvements are still needed to measure vocal fold material properties at phonatory frequencies. However, the results described here are the first steps towards that ultimate goal, and provide a good first order approximation that may be used in phonatory modelling.

CONCLUSION

The vibratory, acoustic, and aerodynamic properties of the larynx are dependent on the neuromuscular anatomy as well as material properties of the vibratory tissue. Thus, models used to study laryngeal physiology and voice production should match these parameters to the human larynx as closely as possible. In this regard, this study measured the Young's modulus of canine and human vocal fold cover layers and found them to be identical. This new information on cover layer material property, combined with previously reported comparison of canine and human laryngeal geometry and neuromuscular anatomy showing close match between the two species, supports the role of the canine larynx as an excellent in vivo model to study human laryngeal physiology.

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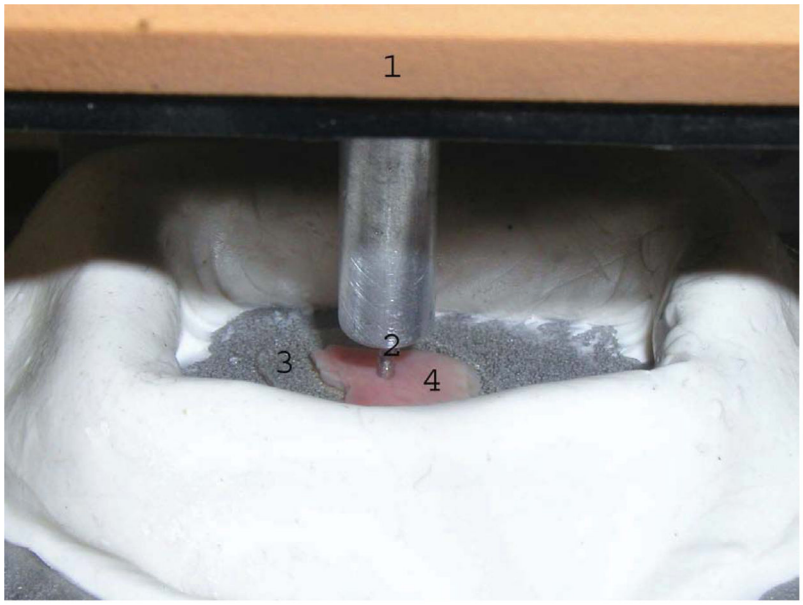

Figure 1.

Indentation apparatus for measuring the elastic modulus of vocal fold cover layers, showing (1) force transducer, (2) indenter, (3) normal saline to keep specimen moist, and (4) vocal fold cover layer specimen.

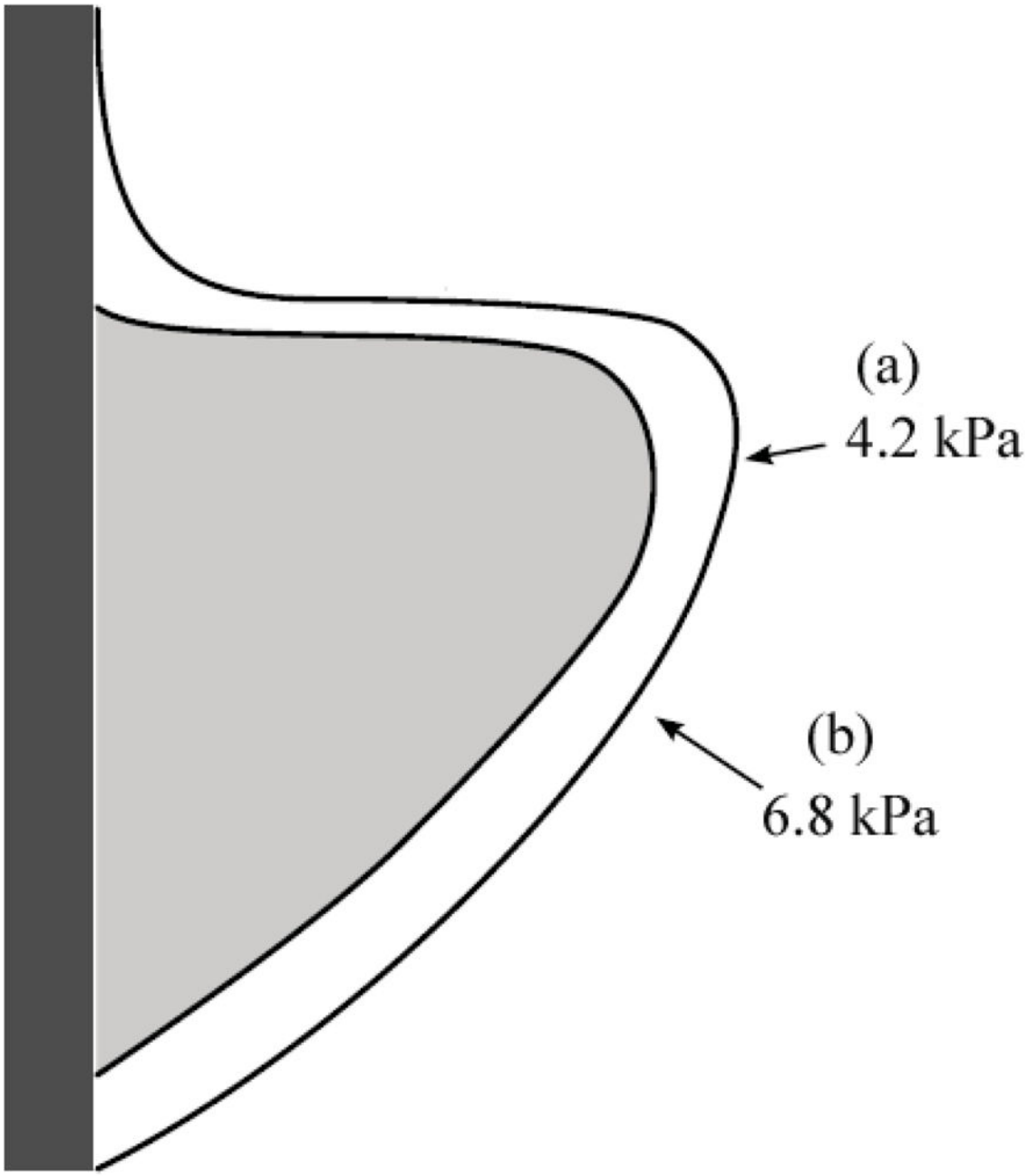

Figure 2.
Coronal section of ex vivo canine vocal fold illustrating the estimated Young's modulus of (a) superior medial surface (superior 3–4 mm of the medial vocal fold surface cover layer) and (b) inferior medial surface (inferior 3–4 mm of the medial vocal fold surface cover layer).

Table 1

Estimated Young's Modulus of Canine and Human Vocal Fold Medial Surface Cover Layers

	Canine Medial Cover		Human Medial Cover	
Sample	**Superior (Pa)**	**Inferior (Pa)**	**Superior (Pa)**	**Inferior (Pa)**
1	5384	6792	5360	7230
2	4645	7057	4680	6671
3	3665	6560		6791
4	5303	8531		7306
5	4206	6190		
6	3032	6382		
7	3433	6733		
8	4248	6587		
9	4310	7096		
10	3940	5397		
11	4206	5995		
12	4190	7634		
13	4867	7236		
14	4327	7910		
15	3976	6821		
16		6830		
17		5613		
Mean	4249	6786	5020	6999
SD	633	788	481	316
Range	3.0 – 5.4 kPa	5.4 – 8.5 kPa	4.7 – 5.4 kPa	6.7 – 7.3 kPa

Pa = Pascals;

kPa = kilopascals