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Use of the rabbit larynx in an excised larynx setup

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Abstract

Objective—To modify the excised larynx bench apparatus to accommodate experiments with rabbit larynges.

Study design—Methodological study using *ex vivo* rabbit larynges

Methods—Rabbit larynges (n=5) were dissected and mounted on a custom-made phonatory apparatus. The arytenoids were adducted by rods and humidified air was passed through the larynx to elicit vocal fold vibration. Acoustic, aerodynamic, electroglottographic, and videokymographic data were collected for each larynx. The same data were collected for 5 canine larynges for the purpose of comparison, and coefficients of variation were calculated for each parameter in both models.

Results—Reliable phonation was achieved in each larynx. Acoustic fundamental frequency, % jitter, % shimmer, signal-to-noise ratio, pressure and flow at phonation onset and offset, and fundamental frequency, closed quotient, speed quotient, jitter, shimmer, and contact quotient, as recorded by electroglottography, and mucosal wave amplitude and phase difference are reported for rabbit larynges. Coefficients of variation for each parameter are similar in magnitude between the two models.

Conclusion—We developed a method for recording reliable acoustic, aerodynamic, videokymographic, and electroglottographic data from rabbit larynges. When data obtained from leporine larynges were compared with data from canine larynges, the intra-larynx variability of rabbit larynges was found to be similar to that of canine larynges.

Introduction

Excised larynges have been used for centuries as models for simulating laryngeal pathologies and to mimic human voice production.^{1–3} The canine larynx is the most commonly used model due to its similarity in size and gross structure to the human larynx; it is also easily manipulated, phonates in a laboratory setting, and exhibits vocal fold vibration similar to what is observed in humans.^{4,5} However, there are several limitations that make extrapolating findings to humans difficult. The vocal fold layers of the canine larynx differ greatly from that of the human larynx. Though both have a three-layered lamina propria, the

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surface layer in humans is mostly ground substance, while dogs have thin sheets of collagen and elastin above loose ground substance in their surface layer. These differences could present difficulties when making comparisons to humans, especially in studies of tissue pathologies such as cysts or scars.⁴ (Figure 1) Dogs also have a poorly defined vocal ligament (intermediate and deep lamina propria).

For these reasons, other animal models, such as pigs, cows, sheep, deer, rats, and rabbits, have been considered in recent years.^{5–8} Of these, the rabbit larynx is particularly interesting as the histology of the rabbit lamina propria is very similar to that of humans.⁹ Humans have a superficial layer of loose ground substance, an intermediate layer composed of dense ground substance and elastin, and a deep layer of mainly collagen.⁴ Rabbits possess a similar superficial layer over an intermediate layer of ground substance and collagen, and a third, deeper layer of collagen.¹⁰ Thus, the rabbit larynx has proven useful for hybrid *in vivo/ex vivo* studies and in histological studies of the vocal fold.^{9,11,12} Additionally, the rabbit is comparatively inexpensive and easy to care for in a laboratory setting.

A number of studies have examined the rabbit larynx in terms of histology and immunological response, especially with regard to vocal fold wound healing and response to surgical implantation, but very rarely has it been used in studies of vocal fold mechanics; those studies have generally been conducted using canine larynges. Swanson et al.¹³ used rabbit models to study the effects of normal phonation and phonotrauma on the extracellular matrix, and Hertegård et al.⁸ used air pulse stimulation to examine the elasticity of scarred rabbit vocal folds. However, studies of mechanisms well-documented in other models, such as arytenoid adduction and vocal fold elongation, are absent for rabbit larynges.

Since canine larynges have been studied extensively, the methods for data collection are firmly established. Rabbit larynges are much smaller than the other models that have been explored in lieu of canine models and larger than rat larynges, which have also been examined as an alternative to canine larynges. Therefore, much of the equipment currently used for data collection on these larynges is inadequate for collection of the same data on rabbit larynges.

In this study, we present modifications to the excised larynx bench apparatus that can be made to accommodate rabbit larynges, a potentially valuable complement to the canine larynx for *ex vivo* studies of phonation. We propose methods for dissection and excision of the rabbit larynx, for mounting the larynx and obtaining reliable phonation, and for collecting acoustic, aerodynamic, videokymographic, and electroglottographic data.

The excised rabbit larynx can be used to accurately and reliably quantify vocal fold vibrations using acoustic, aerodynamic, and glottographic techniques. This model will be useful in experiments altering the tissue properties of the vocal folds since rabbit vocal folds are histologically similar to human vocal folds.

Methods

Excision

Larynges were harvested less than 12 hours post-mortem from adult New Zealand White rabbits (n=5). The rabbits were surplus experimental animals euthanized and donated by Covance, Inc. (Madison, WI). No animals were sacrificed expressly for the purpose of this study. Age and sex information were not recorded for the animals. A midline vertical incision was made from the anterior belly of the digastric to the sternum. The trachea was exposed, and the surrounding muscles and tissue were cut away. The trachea was cut approximately 10 mm below the cricoid cartilage for ease of mounting. A cut was made

through the pharynx 5–7 mm above the larynx, and the attached tissue was cut away to liberate the larynx. The surrounding muscles and tissue remained attached to the larynx until it was prepared for mounting. Larynges were stored for a minimum of 2 days and a maximum of 9 weeks in 0.9% saline and frozen in a -20°C freezer before use. Canine larynges ($n=5$) were harvested from mongrel dogs within 24 hours postmortem from animals sacrificed for non-research purposes. Larynges were excised according to the procedure described by Jiang and Titze.¹⁴ The larynges were stored in 0.9% saline at -20°C until use.

Dissection

Before use, larynges were thawed individually in a room temperature water bath. Remaining excess tissue was carefully trimmed away using surgical microscissors until the cartilage of larynx was free of extraneous tissue. The epiglottis was removed to expose the arytenoid cartilages. Approximately 5 mm of trachea was left below the cricoid cartilage for ease of mounting, and the thyroid cartilage along with any remaining excess tissue was gradually trimmed to expose the true vocal folds (Figure 2). 0.9% saline was used throughout the dissection to hydrate the larynx. Canine larynges were dissected in the same manner, leaving 2–3 cm of trachea below the cricoid cartilage and removing the corniculate cartilage as well.

Mounting

A custom-built mounting apparatus was used in this study and is shown in Figure 3. The larynx was mounted on a Luer lock (Vernier Software and Technology, LLC, Beaverton, OR) attached to the center of the apparatus (Fig. 1, Part E). The rods on either side of the Luer lock were used to adduct the arytenoids. A suture was placed through the thyroid cartilage and fastened to an elongation apparatus to maintain control of vocal fold elongation. The trachea was securely fastened to the Luer lock using a zip tie (McMaster-Carr, Atlanta, GA) and glue (Loctite, Westlake, OH) to prevent air leakage during phonation, and the entire apparatus was mounted on a bench and attached to the air source via Luer lock prior to experimentation. Canine larynges were sutured in the same manner, but secured to a bench-top apparatus with a metal pull clamp around the trachea to prevent air leakage. The arytenoids of the canine larynges were adducted by lateral prongs to achieve vocal fold approximation.

Data Collection

All studies were conducted in a sound-attenuated room. Five trials were collected for each larynx. Air was passed through a two-heater-humidifier system before reaching the larynx. Airflow was under manual control and slowly increased until vocal vibration was initiated. Airflow, pressure, acoustic, and EGG data were simultaneously recorded using a National Instruments data acquisition board (model AT-MIO-16; National Instruments Corp., Austin, TX) and a custom-designed LabView 8.5 program (National Instruments Corp.). Acoustic data were recorded with a Sony microphone (model ECM-88; Sony Electronics Inc., New York, NY) placed at a 45° angle approximately 10 cm away from the vocal folds, posterior to the arytenoid cartilages to minimize noise from turbulent airflow. Aerodynamic data were recorded by a Heise digital pressure meter (901 series, Ashcroft Inc., Stratford, Connecticut) and an Omega airflow meter (model FMA-1601A, Omega Engineering Inc, Stamford, Connecticut) located below the larynx.

Video of the vocal fold mucosal wave was obtained from a superior perspective to the vocal folds using a high-speed digital camera (model Fastcam-ultima APX; Photron, San Diego, CA). For the rabbit larynges, a 90 mm lens with a bellows attachment was used to increase the magnification to an appropriate level. The videos were collected at a rate of 4,000 frames per second (fps), with a resolution of 512×256 pixels and a length of 768 frames each.

Electroglottographic measurements were obtained by placing silver electrodes with a 2 mm diameter on either side of the thyroid cartilage at the level of the vocal folds. Signa-Gel electrode gel (Parker Laboratories, Orange, NJ) was used to facilitate data collection.

The following variables were measured: pressure and flow at phonation onset and offset; acoustic fundamental frequency, percent jitter, percent shimmer, and signal-to-noise ratio; electroglottographic fundamental frequency, shimmer, closed quotient, speed quotient, shimmer, and contact quotient; and amplitude and phase difference of each vocal fold.

Data analysis

High-speed video data were analyzed using a custom MatLab program (MathWorks, 2008). Amplitude of vocal fold vibration was measured in pixels. Kymographs were generated from columns placed at 50% of the midline from anterior commissure to vocal process. Acoustic data were processed and analyzed using GoldWave v. 5.66 (GoldWave Inc, St. John's, Newfoundland) and TF32 (Demo Level). Electroglottographic data were analyzed using a custom MatLab program (MathWorks, 2008). Coefficients of variation for all parameters in each model were calculated.

Results

All results for the rabbit larynges are reported in Table 1. Pressure and flow at phonation threshold were $16.50 \text{ cm H}_2\text{O} \pm 1.24$ and $4.61 \text{ L/min} \pm 0.41$. The mucosal wave amplitudes for the left and right folds were $3.32 \text{ pixels} \pm 2.35$ and $2.92 \text{ pixels} \pm 2.88$, respectively. Acoustic fundamental frequency was $357.76 \text{ Hz} \pm 116.18$, and the acoustic fundamental frequency given by electroglottography was $450.68 \text{ Hz} \pm 21.12$. There was a similar discrepancy in the canine larynges for these parameters: the acoustic fundamental frequency was $227.90 \text{ Hz} \pm 30.29$ while the electroglottographic fundamental frequency was $322.73 \text{ Hz} \pm 3.80$.

Although there were differences in the coefficients of variation between the two models, the reported coefficients of variation for each parameter were similar in magnitude. For example, the coefficient of variation for phonation threshold pressure was 0.08 in the rabbit larynges and 0.09 in the canine larynges, and for shimmer it was 64.07 in rabbit larynges and 95.90 in the canine larynges. Coefficients of variation for all parameters in both models are reported in Table 2.

Discussion

We developed methods to obtain accurate and reproducible data from excised rabbit larynges. We determined appropriate and repeatable methods for dissection, excision, and mounting of the larynx, achieving reliable phonation, and collecting videokymographic and electroglottographic data. The setup we have also allows for adjustable arytenoid adduction and anterior-posterior elongation, which will be useful in future studies on phonation. In addition, we recorded the same parameters using very similar methods in 5 canine larynges to compare the intralarynx variability. (Table 2) Certain parameters, such as mucosal wave amplitude, had fairly large standard deviations, which could be due to small changes in subglottal pressure among the trials of each larynx. However, because both groups of larynges show similar variability, we can conclude that our modified method for data collection from rabbit larynges is as reliable as that for canine larynges.

Canine larynges will most likely prove more useful than rabbit larynges for studies manipulating the gross anatomy of the larynx (e.g., laryngeal muscle activation) but rabbit larynges will be valuable for studies evaluating vocal fold tissue change. The rabbit larynx is

much smaller than the canine larynx, and therefore requires more time and care during dissection and mounting; it is also more difficult to manipulate. However, many of these difficulties are ameliorated by practice, with the difference in time spent dissecting and mounting each type of larynx becoming nearly negligible. The added benefits of a tissue structure similar to humans and a lower cost contribute to the model's usefulness in this particular field of study.

There were several challenges to adapting the excised larynx bench apparatus to rabbit larynges. Various challenges were encountered during pilot testing. The videos for some larynges (not included in this study) were too dark to analyze, which was remedied during the course of the study by placing the lights at a standardized location and angle. There was a discrepancy between the F_0 found via acoustic measurements and the fundamental frequency found from EGG data. This is most likely due to differences in electrode placement and microphone placement. EGG electrodes are sensitive to placement in any larynx, and because the rabbit larynx is so small, any change in location can lead to a change in the data; microphones are also notoriously sensitive to placement and sound differences.¹⁴ In the future, we will develop a method for standardizing electrode placement. The canine larynges also demonstrated a higher fundamental frequency as measured by EGG compared to the fundamental frequency measured by the microphone, and the degree of that discrepancy was comparable to the rabbit larynges.

Further studies that would be of benefit in this area of research would be a multi-species comparison of mechanical parameters among human, canine, and rabbit larynges, as well as a comparison of the laryngeal histology among the three species. In the future, we hope to do studies to evaluate treatments for vocal fold scarring using rabbit larynges by scarring the vocal folds and administering treatments *in vivo*, then excising the larynges for *ex vivo* tests. With this model, we can directly control the scarring and have a larger sample size for each study, rather than doing studies on humans, which can be costly both in time and expense. Using rabbit larynges for such studies can also shed light on the process of scar formation and tissue healing, since the larynges may be excised at different time points.

Conclusions

The rabbit larynx can be used reliably in excised larynx experiments. In addition, the intralarynx variability is similar to that of canine larynges. Due to histological similarities to humans and potential utility in studies performing *ex vivo* analyses of *in vivo* manipulations, the rabbit larynx is a valuable complement to the traditional canine model.

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	Rabbits	Humans	Canines
Superficial lamina propria	Loose ground substance	Loose ground substance	Sheets of collagen and elastin
			Loose ground substance
Intermediate lamina propria	Ground substance and collagen	Dense ground substance and elastin	Ground substance and collagen
Deep lamina propria	Collagen	Collagen	Collagen

Figure 1.

A schematic showing the vocal fold tissue layers in rabbit, human, and canine larynges.

Figure 2.

A: A rabbit larynx dissected to expose the vocal folds. All excess tissue has been cut away, and the epiglottis and excess thyroid cartilage have been removed. **B:** The rabbit larynx is mounted on a custom apparatus using a Luerlock, a zip-tie, and Loctite glue. The arytenoid cartilages are adducted by rods to produce phonation, and adequate anterior-posterior elongation is achieved through the placement of a suture in the thyroid cartilage at the level of the vocal folds. EGG electrodes are attached on either side of the thyroid cartilage at the level of the vocal folds by electrical tape.

Figure 3.

A diagram of the excised rabbit larynx mounting system. **A** shows a rear angle view of the setup. **B** shows a frontal view with both adduction apparatuses and the elongation apparatus viewable.

A= Smooth rod used for manipulation of the arytenoid cartilages, which allows for control of vocal fold adduction levels; the angle was found to be necessary to obtain approximation on the inferior margin of the vocal fold

B= Spring-loaded mounting rods that allow for AP positioning of the threaded rod (A)

C= The base of the system. It has channels to allow for AP movement of the rods used for arytenoid adduction and

D= Symmetric AP elongation apparatus. Suture is tied to the thyroid cartilage at the anterior commissure and connected to this apparatus which can be positioned in the same manner as (B)

E= Plastic mount for the larynx. A portion of the trachea is left and clamped to this mount. This provides stability for the larynx and an entry point for airflow.

F= The tube connector used as an interface between the building source of pressurized air and the phonation system

G= Extra pieces of metal rod used to clamp the phonation system to a laboratory bench. This will be removed once the system is incorporated into a laboratory bench.

Table 1

Summary data for reported parameters of phonation in rabbit larynges (n=5). Data are given as mean $\pm$ standard deviation

	Mean $\pm$ SD
Phonation threshold pressure (cm H ₂ O)	16.50 $\pm$ 1.24
Phonation threshold flow (L/min)	4.61 $\pm$ 0.41
Phonation offset pressure (cm H ₂ O)	15.69 $\pm$ 0.97
Phonation offset flow (L/min)	1.51 $\pm$ 0.36
F0 (acoustic, Hz)	357.76 $\pm$ 116.18
Jitter, % (acoustic)	0.020 $\pm$ 0.018
Shimmer, % (acoustic)	22.07 $\pm$ 14.32
Signal-to-noise ratio	10.11 $\pm$ 2.03
F ₀ (EGG, Hz)	450.68 $\pm$ 21.12
Closed quotient	0.028 $\pm$ 0.0066
Speed quotient	1.09 $\pm$ 0.054
Jitter	5.02*10 ⁻⁵ $\pm$ 0.049
Shimmer	0.0025 $\pm$ 0.63
Contact quotient	0.64 $\pm$ 0.019
Mucosal wave amplitude (right fold, pixels)	2.92 $\pm$ 2.88
Mucosal wave amplitude (left fold, pixels)	3.32 $\pm$ 2.35
Phase difference (right fold, pixels)	0.73 $\pm$ 0.52
Phase difference (left fold, pixels)	0.64 $\pm$ 0.32

Table 2

Coefficients of variation for measured parameters from rabbit larynges (n=5) and canine larynges (n=5).

	Mean Intralarynx Coefficient of Variation	
	Rabbit	Canine
Phonation threshold pressure	0.08	0.09
Phonation threshold flow	0.06	0.11
Phonation offset pressure	0.06	0.09
Phonation offset flow	0.03	0.07
F ₀ (acoustic)	0.33	0.13
Jitter, % (acoustic)	0.81	0.43
Shimmer, % (acoustic)	0.66	0.61
Signal: noise ratio	0.20	0.13
F ₀ (EGG)	0.05	0.01
Closed quotient	0.45	0.30
Speed quotient	0.061	0.04
Jitter	283.68	2.7*10 ⁴
Shimmer	64.07	95.90
Contact quotient	0.031	0.01
Mucosal wave amplitude (right fold)	0.92	0.45
Mucosal wave amplitude (left fold)	0.71	0.42
Phase difference (right fold)	0.61	1.08
Phase difference (left fold)	0.46	0.40