

Optimizing Protein Harvest From Nasal Brushings for Determining Local Allergy Responses

American Journal of Rhinology & Allergy

2018, Vol. 32(4) 244–251

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DOI: 10.1177/1945892418777668

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Abstract

Background: Rhinitis is a highly prevalent yet often misdiagnosed condition. Patients who have local allergic rhinitis are regularly mislabeled as having a nonallergic etiology. Thus, a highly accurate, reproducible, and noninvasive assessment, which can be performed quickly and with minimal discomfort to the patient, is required.

Objective: The aim of this research was to identify the efficiency of various nasal brushes as tools for harvest and collection of epithelial proteins and its suitability for identification of rhinitis.

Methods: Nasal epithelial mucosa samples were taken from patients undergoing turbinate surgery using a cytology brush, a dental brush, and a nasal curette in random order. After washing in phosphate-buffered saline, the suspended cells were sonicated. Total protein content was assessed for all samples by bicinchoninic acid assay measured using a Nanodrop machine. Identification of nasal-specific immunoglobulin E (sIgE) was then assessed using immunoassay and compared to the patient's allergic status from epicutaneous and serum testing. The lower threshold limit for the sIgE in nasal brushings was determined using the results of serum sIgE tests as the reference. The diagnostic accuracy of this new established cutoff value was determined.

Results: The cytology brush was found to be the optimal tool for maximal nasal mucosa protein collection followed by dental brush and nasal curette (0.75 ± 0.45 mg/mL vs 0.43 ± 0.24 mg/mL vs 0.071 ± 0.55 mg/mL, respectively; $P < .01$). The optimal cutoff value of nasal sIgE from the cytology nasal brushings was 0.14 kUA/L to predict allergic status from serum testing. This gave a sensitivity of 75%, specificity of 86%, positive predictive value of 74%, likelihood ratio positive of 5.40, and diagnostic odds ratio of 18.62.

Conclusion: The cytology brush is the optimal tool for protein collection. This is an easy and direct method to sample the nasal mucosa for assessment of nasal allergy or future biomarkers.

Keywords

nasal brush, epithelial proteins, immunoglobulin E, ImmunoCAP, immunoglobulin, noninvasive assessment, rhinitis, total protein

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Introduction

Typically, allergic rhinitis (AR) is diagnosed through clinical symptoms as well as measurement of atopy status by serological detection of specific immunoglobulin E (spIgE)¹ and/or skin prick test (SPT) to measure systemic allergy.² There is mounting evidence to show that some rhinitis patients who have no indication of allergy through these systemic tests, in fact do have localized IgE-mediated disease in the nose.^{3,4} This is termed as local AR and affects up to 70% of patients diagnosed with nonallergic rhinitis (NAR) through conventional methods.^{5–7} Due to the high incidence of local rhinitis and misdiagnosis based on clinical symptoms, a minimally invasive and affordable method is required to accurately differentiate the various etiologies of rhinitis.

Noninvasive methods to assess the nasal mucosa such as nasal lavage, nasal swab, and nasal brushing have been previously proposed. However, nasal brushing is a good option, as it is relatively noninvasive, painless, and tolerable, yet allowing accurate epithelial sampling from the inferior turbinate. The aim of this study was to compare the ability of brushes currently used in other medical fields and to collect total protein from nasal epithelial sampling. The methodology of processing the nasal harvest was also optimized for its reproducibility and suitability to further detect nasal specific spIgE. Future applications might include other biomarkers in chronic rhinosinusitis.

Methods

A cross-sectional study was performed to compare 3 methods of nasal brushing: a cytology brush (EndoscanPlus, Medico, Melbourne, Australia), dental brush (Pikster®, size 7, Erskine Oral Care, Macksville, Australia), and Rhino-pro® Nasal Mucosa Curette (Rhino-Pro®, Arlington Scientific, Inc, Springville, UT) (Figure 1). All participants in this study had symptoms of at least nasal obstruction and were undergoing turbinate surgery. Inclusion criteria required patients to be over 18 years of age and able to give consent. Patients with known systemic conditions, for example,

immunodeficiency, vasculitis, cystic fibrosis, or granulomatous disease, thought to affect nasal mucosa were excluded. Recruitment was performed between September 2015 and April 2016. Appropriate ethics approval was obtained prior to the study (HREC/15/SVH/24), and patients provided informed consent.

Allergy Assessment

Participants were subjected to both serological assessment and epicutaneous testing to determine their allergy status. Serum samples were collected at the time of surgery and assessed for the presence of spIgE against 4 types of allergen mixes (grass, house dust, mold, and animal epithelium) by ImmunoCAP (Phadia 250, Thermo Fischer Scientific Inc., Phadia AB, Uppsala, Sweden) analysis (Table 1). A value equal or above 0.35 kU/L for any 1 of the allergen spIgE mix was defined as a positive allergy status. This is the most commonly accepted laboratory lower threshold for serum spIgE to define a positive test in serum or plasma.⁸

The SPT was performed within 6 months of surgery and was assessed for sensitization to equivalent allergens to the ones in serum (Table 1). The allergens were applied to the patients in a 50% glycerin mixture (Hollister, New York, NY and Western Allergy, Victoria, Canada) using the Multi-Test® II device (Lincoln Diagnostics, Decatur, IL). A negative glycerin control and positive histamine control were used. A skin wheal of 3 mm or more was used to determine a positive response.

Patients with both negative serology and SPT were grouped as nonatopic, while positive results for either tests classified patients as allergen sensitized. Epicutaneous testing for other allergen group (such as tree or weed pollen) was also considered when determining the allergy status.

Sampling

All patients were subjected to mucosal brushings to both nasal cavities utilizing the 3 brushes being evaluated (cytology brush, dental brush, and Rhino-Pro® Nasal

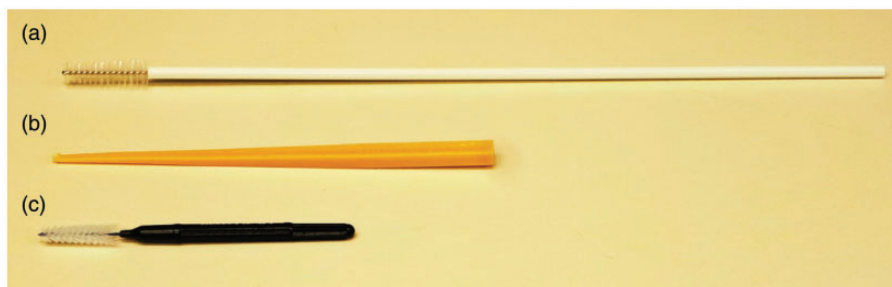


Figure 1. Brushes utilized for mucosal sampling in the nose. (a) Cytology brush, (b) nasal mucosal curette, and (c) dental brush.

Table 1. The List of Allergens Tested in (a) Serum, (b) Skin Prick Test, and (c) Nasal Brushings.

Allergen Groups	(a) Serum	(b) Skin Prick Test	(c) Nasal Brushing Samples
Grass	<i>Lolium perenne</i> (rye), <i>Phleum pratense</i> (Timothy), <i>Poa pratensis</i> (Kentucky blue), <i>Sorghum halepense</i> (Johnson), <i>Paspalum notatum</i> (Bahia), <i>Cynodon dactylon</i> (Bermuda)	5-Grass mix: <i>P. pratense</i> (Timothy), <i>Festuca eliator</i> (meadow), <i>L. perenne</i> (rye), <i>Anthoxanthum odoratum</i> (sweet vernal), and <i>Dactylis glomerata</i> (cocksfoot). <i>L. perenne</i> (rye), <i>P. notatum</i> (Bahia), <i>C. dactylon</i> (Bermuda)	<i>L. perenne</i> (rye), <i>P. pratense</i> (Timothy), <i>P. pratensis</i> (Kentucky blue), <i>D. glomerata</i> (cocksfoot), <i>Festuca pratensis</i> (meadow fescue), <i>P. notatum</i> (Bahia)
Dust	<i>Dermatophagoides pteronyssinus</i> , <i>Dermatophagoides farina</i> , <i>Blattella germanica</i> (German cockroach)	<i>D. pteronyssinus</i> , <i>D. farina</i> , cockroach mix	<i>D. pteronyssinus</i> , <i>D. farina</i> , <i>B. germanica</i> (German cockroach)
Mold	<i>Penicillium chrysogenum</i> , <i>Cladosporium herbarum</i> , <i>Aspergillus fumigatus</i> , <i>Alternaria alternate</i>	<i>Penicillium Cladosporium</i> sp. Mix (<i>Cladosporium cladosporioides</i> , <i>C. herbarum</i>), <i>Aspergillus</i> sp. mix (<i>A. fumigatus</i> , <i>Aspergillus nidulans</i> , <i>Aspergillus niger</i>), <i>A. alternate</i>	<i>P. chrysogenum</i> , <i>C. herbarum</i> , <i>A. fumigatus</i> , <i>A. alternate</i>
Animal epithelium	<i>Felis domesticus</i> (cat), <i>Equus caballus</i> (horse), <i>Bos taurus</i> (cow), <i>Canis familiaris</i> (dog)	<i>F. domesticus</i> (cat), <i>C. familiaris</i> (Dog)	<i>F. domesticus</i> (cat), <i>E. caballus</i> (horse), <i>B. taurus</i> (cow), <i>C. familiaris</i> (dog)

^aGrass allergens varied between serum and nasal brushing due to the different commercially available allergen mix.

Curette) which were applied in a computer randomized order. The nasal brushings were performed, while the patients were under general anesthesia prior to surgery and nasal preparation. The first brush was then inserted on the medial surface of the anterior third of the inferior turbinate and septum. The brush was rotated and moved back and forth 5 times across the inferior turbinate and 5 times across the nasal septum. This was repeated with the same brush on the opposite nostril. After removing the brush, the head was cut off into a cryovial tube filled with 2 mL of phosphate-buffered saline (PBS) in order to extract the mucus and epithelial cells. This procedure was repeated for the other 2 brushes. Samples (containing the brush heads) were frozen immediately at -180°C in vapor phase liquid nitrogen.

Measurement of Total Protein

Samples were thawed at 37°C for 2 min. Brush heads were agitated in PBS for 10 s and scraped along the cryovial edge 5 times while twisting before being removed. Nasal epithelial suspensions were split into duplicate groups and maintained at 4°C .

All samples were sonicated on ice using an ultrasonicator (Ultrasonic processor UTR200 sonoreactor, Hielcher, Germany) to lyse the cells. Sonication was done at 45% amplitude, 10 s bursts, with 10 s intervals for 2 min until the samples appeared clear and no more viable cells were detected by trypan blue staining (Thermo Fischer Scientific, Trypan Blue 0.4%, Waltham, MA). One set of the duplicates was then centrifuged at 4°C , 3000 rpm, for 30 min.

Total protein was measured in all samples supernatants using a bicinchoninic acid (BCA) assay (Thermo Fischer Scientific, Pierce BCA Protein Assay), following the manufacturer's recommendations and absorbances read using a Nanodrop machine (Thermo Fischer Scientific, Nanodrop 1000) measuring at 560 nm wavelength. The remaining samples were stored at -80°C .

ImmunoCAP Analysis

The cytology brushing samples were sent for analysis to determine the concentration of total IgE and sIgE (for all 4 allergen groups). Specimens were left to defrost naturally and analyzed using an ImmunoCAP machine (Phadia 250), following the standard protocol for serum testing set by the laboratory. Equivalent sIgE to the serum was tested and expressed as kUA/L (Table 1). The lowest detection limit was 0.01 kUA/L.

Statistical Analysis

All statistics were performed using SPSS version 24, and graphs were also generated with the same software. The total protein content from each type of brushings was compared using a repeated measures analysis of variance with a Bonferroni post hoc analysis. An independent *t* test was used to compare allergy status with protein levels and total IgE collected by the cytology brushes. Paired samples *t* test was performed to check for significant differences in the presence or absence of centrifugation. All results were given as mean $\pm$ standard deviation. Pearson correlation coefficient was used to compare cytology brushings total IgE with serum total

IgE and also to compare cytology brushings total IgE with cytology brushings protein concentration.

A receiver operating curve (ROC) was generated to determine the best cutoff value for nasal allergen spIgE using its corresponding serum allergen spIgE as reference test. This was applied to all of allergen spIgE mix groups (grass, dust, mold, and animal epithelium). Each allergen spIgE in nasal brushings was paired with its serum group counterpart (resulting in 4 sets per patient). The ROC was then applied to this combined group. The sensitivity, specificity, likelihood ratio positive (LR+), positive predictive value (PPV), and diagnostic odds ratio (DOR) was determined using the determined cutoff. This was compared to the traditional cutoff value of more or equal to 0.35 kUA/L. The study was powered to detect 25% difference in protein concentration between the brushes group.

Results

There were 47 patients who participated in this study, age (40.95 ± 15.67), 36% females, 63.8% had asthma, and 76.6% were allergen sensitized. Grass, dust, mold, and animal epithelium sensitization (either positive SPT/serum) occurred in 57.4%, 57.4%, 21.2%, and 36.1%, respectively. Among the allergen sensitized, 72.2% were sensitized to more than 1 allergen group. Other allergen

group sensitization (tree or weed pollen) from epicutaneous testing occurred in 27.6%. However, these patients were also sensitized to at least 1 of the 4 main group of abovementioned allergens.

Total Protein Concentrations Between Brush Types

Total protein concentration was significantly different between cytology brush, dental brush, and Rhino-Pro® Curette (0.75 ± 0.45 mg/mL vs 0.43 ± 0.28 mg/mL vs 0.07 ± 0.55 mg/mL, $P < .01$) with a Wilks' lambda with value of 0.27. Each of the brushes was significantly different (Figure 2). As the cytology brush gave the highest protein yield, further analyses were represented by the cytology brush.

Centrifuge Versus Noncentrifuged Samples

There was no significant difference between protein concentration of raw (noncentrifuged) samples and the centrifuged samples harvested using the cytology brush (0.76 ± 0.44 mg/mL vs 0.75 ± 0.45 mg/mL, $P = .18$).

Protein Concentrations and Allergy Status

There was no significant difference in protein concentration between the nonatopic and allergen sensitized group

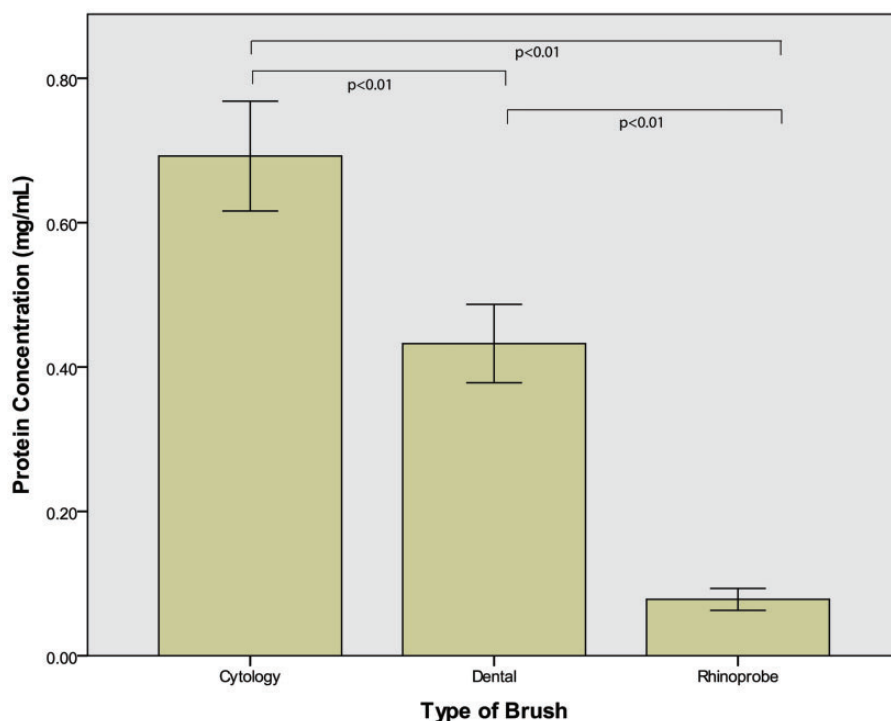


Figure 2. Protein yield for each sampling technique. Mucosal brushing samples collected utilizing the cytology brush, dental brush, and Rhino-Pro® were processed to obtain a total protein yield, measured using a Nanodrop. Data are represented as mean protein concentrations $\pm$ 95% CI (error bars) and were assessed for statistical significance using a Wilks' Lambda and Bonferroni post hoc test.

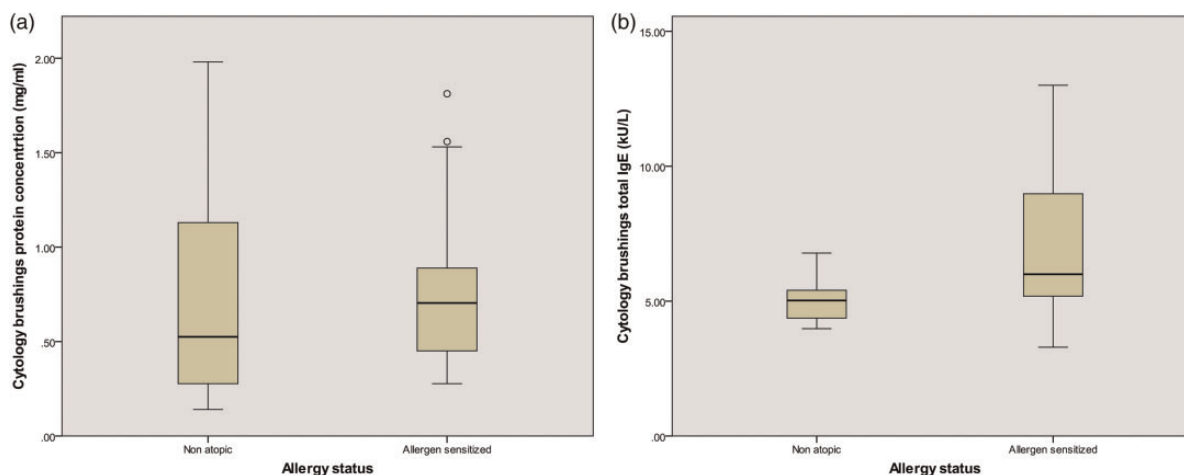


Figure 3. Comparison of the cytology brushings content between the nonatopic and allergen sensitized patient groups. (a) Comparison of the protein concentration and (b) total IgE from the cytology brush between nonatopic and the allergen sensitized patients. Patients were grouped based on their diagnosed allergic status. Data are represented as mean concentration values $\pm$ 95% CI. Statistical analysis was performed using an independent *t* test.

using the cytology brush (0.75 ± 0.62 mg/mL vs 0.75 ± 0.39 mg/mL, $P = 0.98$) (Figure 3(a)).

Cytology Brushings Total IgE

The mean total IgE for the nasal cytology brushing was 6.56 ± 2.57 kU/L. This was lower in the nonatopic population compared to the allergen sensitized (5.03 ± 0.81 kU/L vs 7.05 ± 0.74 kU/L, $P < .01$) (Figure 3(b)). There was no correlation between cytology brushings total protein and cytology brushings total IgE ($r = -.12$, $P = .45$). There was a moderate positive correlation between total IgE in nasal brushings and total IgE in serum ($r = .35$, $P = .03$).

Cytology Brushings Specific IgE

The mean nasal sIgE for each allergen mix was grass mix: 0.22 ± 0.33 kUA/L, dust mix: 0.35 ± 0.54 kUA/L, mold mix: 0.16 ± 0.15 kUA/L, and animal epithelium mix: 0.12 ± 0.07 kUA/L.

The ROC gave a positive cutoff of 0.14 kUA/L for the cytology nasal brushings (area under the curve: 0.871, 95% confidence interval: 0.809-0.933, $P < .01$) (Figure 4). This gave a sensitivity of 75%, specificity of 86%, PPV of 74%, LR+ of 5.40, and DOR of 18.62. When the traditional (serum) positive cutoff of 0.35 kUA/L or more was used for the nasal brushing samples, it gave a sensitivity of 35.71%, specificity of 100%, and PPV of 100% (LR+ and DOR could not be calculated). The 2 by 2 table is presented in Table 2.

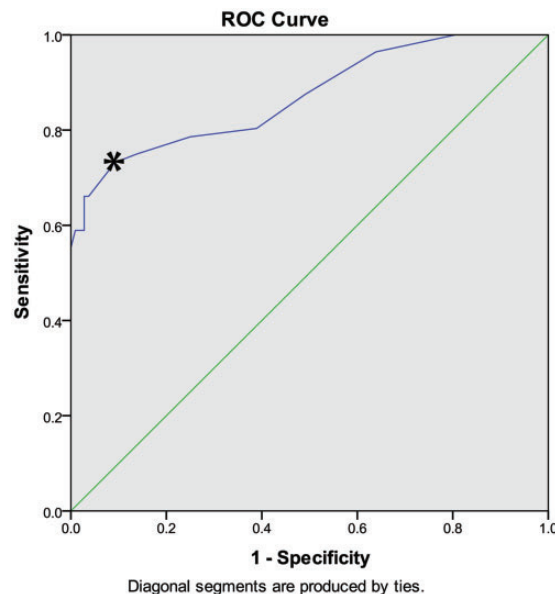


Figure 4. Receiver operating curve (ROC) for nasal cytology brushing sIgE to determine allergy status. The serum sIgE of the corresponding allergen was used as the reference test. A cutoff level of 0.14 kUA/L(*) of the sIgE from the nasal cytology brush gave a sensitivity of 75% and specificity of 86%.

Discussion

Comparison between cytology brush, dental brush, and Rhino-Pro[®] Curette revealed that the cytology brush was the most effective tool for collection of nasal proteins from the nasal mucosal surface.

The potential differences could be due to their structural features. The cytology brush had a longer handle which gave easier access to the site of interest and helped

Table 2. Two by Two Tables Comparing Cytology Brushings spIgE With Serum spIgE as Reference.

		Serum spIgE		Total
		Positive	Negative	
(a) Cytology brushing spIgE positive cutoff ≥ 0.14 kUA/L				
Cytology	Positive	42	15	57
brush spIgE	Negative	14	93	107
	Total	56	108	164
(b) Cytology brushing spIgE positive cutoff ≥ 0.35 kUA/L				
Cytology	Positive	20	0	20
brush spIgE	Negative	36	108	144
	Total	56	108	164

Two cutoff measures were used (a) equal or more than 0.14 kUA/L and (b) equal or more than 0.35 kUA/L.

Abbreviation: spIgE, specific immunoglobulin E.

in scraping more required nasal mucosa. The Rhino-Pro® Curette in contrast was designed for collection of cells scrapings for light microscopy testing rather than large amounts of exudate and particulate matter on the surface of the mucosa to be diluted in fluid. The use of 3 different brush types was in contrast to a prior study, which compared the ability to harvest cells specifically, rather than the IgE and protein present on the mucosa and mucosal secretions.⁹ Results of that study showed that from a homemade cytology brush, nasal swab, and nasal curette, all were able to sufficiently isolate and allow the culturing of human cells. The cytology brush was nevertheless the best tool used, similar to the results in this study.

Comparison of total protein yield alone was shown to be incapable of differentiating an allergic from a non-allergic population (0.747 ± 0.620 vs 0.752 ± 0.393 mg/mL, $P = .98$). This was likely due to the abundance of other proteins such as immunoglobulin A, M, and G (IgA, IgM, and IgG). Early research did not find any difference between albumin, IgA, and IgM content in nasal secretion between asthmatic patients and the healthy children. However, there was a difference in total IgE content.¹⁰ Similarly, we also found a higher yield of total IgE in the nasal brushings among the allergen sensitized patients (5.04 ± 0.81 vs 7.05 ± 0.74 mg/mL, $P < .01$) despite the similar protein content. However, nasal spIgE is deemed to be more useful to diagnose AR,¹¹ and the nasal brushing method using the cytology brush described was able to detect presence of nasal spIgE. These epithelial suspensions could also be potentially suitable for measurement of inflammatory mediators such as eosinophilic cationic protein¹²; interleukin-5¹³; nasal nitric oxide¹⁴; tryptase¹⁵; and eotaxin-1, eotaxin-2, and eotaxin-3 in the future.¹⁶ This would be useful to assess nasal inflammation at the target organ itself and phenotyping of rhinitis and other conditions.

The use of nasal brushing for the diagnosis of nasal allergy is relatively easy, requires minimal cooperation, and allows diagnosis of local AR. Studies have proven that patients with negative systemic allergy may still react to allergen intranasally.^{17,18} Therefore, a local nasal assessment is attractive to confirm allergy. Alternative methods of noninvasive nasal mucosa sampling have been used. These are nasal washings,¹⁹ blown nasal secretions,²⁰ nasal swab,²¹ or in situ nasal incubation using specialized nasal strips.²² These methods have been proven effective in sampling the nasal mucosa but require more preparation and need specialized equipment which may not be readily available. Furthermore, only secretions could be sampled. Therefore, we chose to follow the mucosal brushing method described by Reisacher.²³ Based on this method, 1 mL of the nasal brushing samples were sent to a commercial lab for ImmunoCAP analysis to determine the amount of spIgE. This sample must be centrifuged since it requires no particulate matter to pass through the machine. Accordingly, it was similarly performed within the protocol devised for this study, in order to see whether there would be an effect on the total protein present, with the results showing an insignificant difference.

The cytology brush was able to detect presence of nasal spIgE. However, determining a positive cutoff to define a positive result remains a challenge. Using the corresponding serum spIgE as a reference standard, we defined a cutoff of ≥ 0.14 kUA/L which gave a sensitivity of 75% and specificity of 86%. This was in contrast to the cutoff value of 0.35 kUA/L which had 100% specificity but poor sensitivity. This traditional cutoff value is commonly used in clinical practice to determine presence of serum spIgE. However, this value was determined based on first generations serum tests when the lower level of detection was limited.²⁴ Current tests with the ImmunoCAP system is able to reliably detect spIgE at 0.1 kU/L. Reisacher and Bremberg²⁵ also performed nasal brushings using a cytology brush and tested for nasal spIgE in a case series of 20 patients diagnosed as NAR. They used a cutoff of 0.1 kUA/L and found 100% of NAR patients were positive for nasal spIgE. All of the nasal spIgE measured were between 0.1 to 0.35 kUA/L. Another study by Gelardi et al.²⁶ used a cutoff point of 0.17 kUA/L. This study sampled for nasal spIgE using the Rhino-Pro® Nasal Curette and used healthy controls as reference test. However, they found more positive nasal spIgE among the control population (AR vs NAR vs healthy control: 73 vs 25 vs 43%, $P = .04$). Reisacher²⁷ also found high positivity among the healthy controls (95%) at the cutoff value of 0.1 kUA/L.

Based on this protocol, 0.14 kUA/L was determined as the threshold to predict allergic sensitization. This was based on serum spIgE results (as reference test) and potentially does not predict a local AR due to local

IgE production in the nasal mucosa itself. However, the threshold value of nasal spIgE in nasal brushings, to predict allergy, is believed to be much lower than the traditional value in serum (0.35 kUA/L). When Reisacher et al. assessed nasal brushing spIgE, utilizing a 0.35 kUA/L threshold, only 75% of AR patients²³ and none of the NAR²⁵ patients had positive test. The 0.35 kUA/L threshold lacks sensitivity, as local nasal allergen responses are usually observed in 86% of AR and 25% of NAR populations.¹⁸ The diagnostic characteristics of nasal brushings to other local assessments, such as turbinate tissue spIgE, to define a more sensitive threshold for nasal spIgE is an area of ongoing research. In this study, patient-specific data for all spIgE allergen groups (serum or nasal brushing) were incomplete for 8 participants due to varying lab protocols (omission of testing for certain allergens) or in reporting test results (reported as ordinal classification).

Conclusion

Cytology brushes represented the optimal tool for protein collection from nasal mucosa. This method of assessment would enable more accurate diagnosis of rhinitis which will enable optimal patient diagnosis and treatment based on etiology. Their validity in detecting nasal spIgE, or other biomarkers in rhinitis and chronic rhinosinusitis, is likely to provide future directions for individualized or personalized treatment strategies.

Authors' Note

Appropriate ethics approval was obtained for this study from St. Vincent's Hospital Ethics Research Committee (HREC/15/SVH/24) and study participants provided informed consent.

Acknowledgments

The author(s) would like to thank research colleague Sophia Ma for help with laboratory work. Also, the laboratory technicians and Kate McGill who performed the pilot tests for the Immunocap analysis.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Richard Harvey is a consultant with Medtronic, Olympus, and NeilMed pharmaceuticals. He has also been on the speakers' bureau for Glaxo-Smith-Kline, Seqirus, and Astra-Zeneca. Janet Rimmer has honoraria with Sanofi Aventis, Novartis, Mundipharma, BioCSL, GSK, Stallergenes. Jessica Tattersall is on the speakers Bureau for Bayer, Seqirus, and Meda. She is also on the advisory board for Seqirus. All other authors have no financial disclosures or conflicts of interest.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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