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# Nasal cytologies – impact of sampling method, repeated sampling and interobserver variability*

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SUMMARY

*The influence of sampling technique, repeated sampling and the interobserver variability on quantitative analysis of nasal cytologies were explored. Nasal cytologies from 12 healthy and 4 allergic volunteers were repeatedly collected with a total of three different sampling techniques. Cells were demonstrated by May-Grünwald-Giemsa staining and by immunostaining for CD45 and eosinophil peroxidase (EPO). To determine the interobserver variability specimens were evaluated by three observers with various levels of nasal cytology experience. When adjusted for the variability due to sampling method and repeated sampling, the staining index for CD45+ cells was 53.4 ± 11% (interindividual coefficient of variation: 0.21) with an average intraindividual coefficient of variation of 0.14. For CD45, staining indices differed significantly between the washing techniques and the nasal swabs ($p<0.01$). Repeated sampling did significantly influence the staining indices of specimens collected with nasal swabs ($p<0.05$). Subjects with nasal allergy had less CD45+ cells in their nasal cytologies than the non-allergic subjects ($p=0.02$). A high level of compliance for the quantitative analysis of nasal cytologies between three observers was found ($\kappa_w$ range 0.95-0.98). For studies that require repeated sampling, the lavage will be recommended as best suitable to obtain nasal cytologies.*

*Key words: nasal cytology, interobserver variability, repeated sampling, nasal lavage, quantitative analysis*

## INTRODUCTION

Nasal cytologies provide valuable information about nasal physiology and pathophysiology. However, nasal cytologies are subject to considerable intra- and interindividual variability. This variability is in part due to intrinsic diurnal variations, individual exposure to environmental pollutants [1], intercurrent subclinical infections and the nasal allergy status. In addition to these individual factors, results of nasal cytological examinations are influenced by technical factors including the sampling technique [2] sampling repetitions and the experience of the observer.

To retain cells from the upper airway mucosa, numerous sampling techniques have been described [3,4] in fact, the most natural way to obtain nasal secretions is blowing the nose. However, the amount of secretions obtained by nose blowing is frequently insufficient for reliable examinations [5]. To overcome the scarcity of secretions obtained, approximately 100-200 μl isotonic solution can be sprayed into the nasal cavity with metered dose nebulizers. After a few seconds, sufficient amounts of slightly diluted nasal secretions can be obtained by nose blowing. With nasal lavages, approximately 3-5 ml isotonic fluid is warmed to 37°C, instilled into the nasal cavity, and subsequently recovered. Cells suspended in the recovered lavage fluid can be evaluated [3]. Best results for preservation of cellular morphology were obtained with lactated Ringer's solution as lavage fluid [6]. Swab and brushing techniques involve the insertion of a sampler with adsorptive properties, such as cotton swabs, plastic strips, polyurethane foam samplers or cytology brushes. Following harvest, the cells are detached from the sampler for evaluation [7].

Occasionally, only single sampling is sufficient but often repeated sampling is required, i. e. before, throughout and after a work shift in investigations of occupational hazards, short term exposure settings or a certain treatment [8]. However, intra subject variability in nasal secretions of lavage samples obtained at three different times of a day ranging between 40% and 60% was observed [9]. This variability is presumably biased by the short period between two sampling times. To avoid this bias, and to achieve reproducible baseline levels the sequential nasal lavage with preceding prewashes was established [10].

In general, preparations of nasal cytologies are inhomogeneous and contain various decayed cell elements, debris and highly viscous mucus strands [11]. This heterogeneous composition of nasal specimens aggravates visual examinations. The variability between several observers evaluating nasal cytologies

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has not been reported in the literature. Some studies deal with the interobserver variability on other fields. A high level of interobserver agreement between two observers was found for the histological diagnosis of chorioamnionitis [12]. Good interobserver agreement in classifying squamous metaplastic lesions was found in stained smears of the uterine cervix [13].

In this study the influence of a) three sampling methods, b) repeated sampling, c) the allergic status and d) interobserver variability of three observers with different levels of nasal cytology experience should be evaluated. Because the respective procedures can affect themselves with allergic subjects differently than with nonallergic subjects, both allergic and nonallergic subjects were enclosed, in order to examine for potential interactions. The investigations were accomplished with the following parameters. Cell viability was used to discriminate viable cells from detritus and degranulated cell elements. To differentiate leukocytes from epithelial cells, specimens were stained with CD45. CD45 labels almost all leukocytes whereas epithelial cells were CD45 negative. Hence the ratio of leukocytes and epithelial cells was calculated. The distribution of neutrophils and eosinophils was from interest concerning the composition of leukocyte cells.

## MATERIALS AND METHODS

### *Study population and design*

Sixteen non-smoking volunteers aged 21 to 38 years participated in this study. Twelve were healthy, non-allergic, and free of acute or chronic nasal disorders. In addition, 4 otherwise healthy participants with seasonal grass pollen allergy and no other allergies were selected. In all participants, a detailed clinical history was evaluated and a standard prick test to a panel of common Central European aeroallergens [14] was performed. The study was arranged outside the grass pollen season. None of the participants received topical or oral corticosteroids or any other nasal or anti-allergic medications for at least six weeks before the study. Each subject gave its written consent to participate in the study. Three sampling techniques were employed: a nasal spray-blow technique, a nasal lavage, and a swab technique using polyurethane foam samplers (swab). Each technique was performed at each subject at one examination day at three times of the day: 8:00, 8:30 and 16:00. At least four days were left between each examination day to reduce carry-over effects.

### *Sampling techniques*

Nasal spray-blow technique: 200 µl of prewarmed sterile lactated Ringer's solution was sprayed in each nasal cavity using a nasal pump metered dose applicator (Allergopharma, Reinbek, Germany), which emitted 50 µl per actuation. The droplet-size ranged between 30 and 50 µm. During application the participant reclined his head and held his breathe. Ten seconds after spray application, the participant forcefully blew out the nose into a glass dish. The sample was placed on ice and centrifuged (1200 rpm, 10 min, 4°C). The supernatant was discarded and the cells were resuspended in 0.2 ml of ice cold lactated Ringer's solution.

Nasal lavage: 5 ml of prewarmed sterile lactated Ringer's solution were instilled into each nostril with a syringe. Volunteers were asked to hold their head back and to close the nasopharynx by saying "K". After 10 seconds, the volunteer blew out his nose into a glass dish. Specimens were chilled on ice. After centrifugation cells were resuspended in 0.2 ml of ice cold lactated Ringer's solution.

Polyurethane-foam-sampler (swab) technique: Open cell flexible polyurethane foam samplers in squares of 28 x 18 x 6 mm were placed into each nasal cavity posterior to the mucocutaneous junction and left for 10 minutes [9]. Following removal, the samplers were centrifuged on a piston of a syringe within a polystyrene tube. The supernatant was discarded and the obtained cells were resuspended in 0.1 ml ice cold lactated Ringer's solution. To remove adherent cells, samplers were washed with 5 ml ice cold lactated Ringer's solution while pushing them gently on a cell sieve (mesh diameter 0.3 mm). The obtained solution was centrifuged and the cell pellet was resuspended in 0.1 ml ice cold lactated Ringer's solution. Finally, both cell suspensions were pooled resulting in a final volume of 0.2 ml cell suspension.

### *Viable cell count, cytocentrifugation and storage*

For cell count and viability determination, a 0.01 ml aliquot of the final suspension was mixed with an equal volume of 0.4% trypan blue (Sigma, Deisenhofen, Germany) and assessed in a Neubauer hemocytometer (Marienfeld, Bad Mergentheim, Germany). Cells were then cytocentrifuged on slides with the Zyto-System (Heraeus, Hanau, Germany, 600 rpm, 10 min, 4°C) in a density of 200 vital cells/mm$^2$ on a sedimentation area of 50 mm2. Slides were air-dried and fixed in pure acetone for 10 minutes, wrapped in aluminium foil and stored at -80°C until staining.

### *May-Grünwald-Giemsa staining*

One hundred forty-four (16x3x3) specimens were stained with the May-Grünwald-Giemsa (MGG) method. After thawing, slides were transferred to May-Grünwald solution for 4 minutes and rinsed with water. Subsequently slides were immersed to Giemsa solution (3%) for 15 minutes, rinsed once more and air-dried.

### *Immunocytochemical staining*

One series of slides (16x3x3=144) was immunocytochemically stained with Leukocyte Common Antigen (LCA) (Dako, Hamburg, Germany). LCA is a monoclonal antibody (mAb) against CD45 and labels the cell membrane of almost all leukocytes. A second series of slides (144) was immunocytochemically stained with an anti-human Eosinophil Peroxidase antibody (AHE-1) (Becton Dickinson, Heidelberg, Germany).

AHE-1 is a mAb against eosinophil peroxidase (EPO), a granule protein specific to eosinophils. The slides were washed in Tris buffered saline (TBS), preincubated with 3% $H_2O_2$ in TBS (20 min, room temperature) to inactivate endogenous peroxidases, blocked 10% swine serum (Dako, Hamburg, Germany) in TBS (30 min, RT), and incubated with 200-fold dilutions of mAb (4°, over night). Reaction products were visualized using an APAAP based kit-system (System 40, Dako, Hamburg, Germany) with naphtol AS-MX/fast red as dye reagent. Positive cells were recognized by their red colour. Finally, sections were counterstained with Mayer's hematoxylin (10%, Sigma, Deisenhofen, Germany) for 30 seconds, washed in tap water, and mounted in glycerin-gelatin solution. Specimens from tonsillar tissue served as positive controls. To evaluate nonspecific staining, 1% bovine serum albumin in TBS without the primary antibody was used as a negative control.

*Evaluation of specimens*

Three hundred cells per slide were evaluated at a magnification of x400 under a CX40 light microscope (Olympus, Hamburg, Germany) [15]. In MGG stained specimens neutrophils and lymphocytes were identified, in immunostains CD45+ and EPO+ cells were identified. A staining index was calculated as the ratio of identified cells per one-hundred counted cells.

*Analysis of interobserver variability*

From the pool of 288 immunocytochemically stained slides, 32 slides were randomly chosen. Thirty-two uniform random numbers ranging from 1 to 288 were generated using Systat 10.2 (Systat Corp., IL, USA). Slides with matching numbers were chosen for analysis of interobserver variability. Specimens were evaluated by three blinded investigators. They were trained on basis of an internal laboratory standard operating procedure for the visual evaluation of nasal cytologies.

*Statistical analysis*

A multilevel regression model for longitudinal data [16] was employed to analyze the logarithms of staining indices obtained with the three sampling techniques at the three time points. Participants were modelled as level-2 units and sampling methods and time points as fixed effects. In addition, the interaction of sampling-method•time-point was calculated. The allergy status was modelled as a random effect. Staining indices as predicted by this model are provided with their standard errors of mean.

As a measure of interobserver variability, Cohen's weighted kappa ($\kappa_w$) for each pair of examiners was calculated. Calculations were performed using Systat 10.2 (Systat Corp., IL, USA) and StatXact Ver. 4.1 (Cytel Software Corp. Cambridge MA, USA).

RESULTS

For each participant, samples for nasal cytologies were obtained with the spray-blow, nasal lavage and nasal swab technique at three times per day.

*Cell counts*

Cell viability was examined for each sample. Overall, 1.9 x $10^5$ viable cells/ml were obtained. Cell viability was not significantly influenced by the sampling method, sample repetition or allergy status. If corrected for the variability due to sampling method, sample repetitions and allergy, 53 ± 3% (mean ± SEM) of identified cells were CD45+. Adjusted individual staining indices ranged from 29% to 75% (Figure 1). Overall, 33 ± 3% of all cells obtained were neutrophils, suggesting that they account for approximately 70% of CD45+ cells in nasal cytologies. Only few lymphocytes were identified in May-Grünwald stains. The overall relative lymphocyte count was 1.5 ± 0.2%. Their relative count was not significantly influenced by the sampling method, repeated sampling or the allergy status.

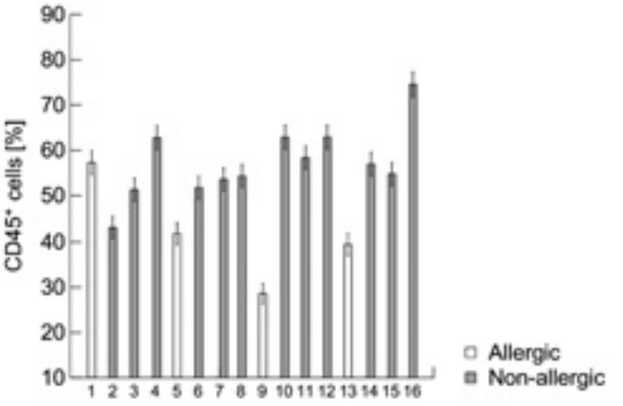


Figure 1. CD45+ cells [%] in nasal cytologies of the 16 participants (x-axis). Each bar represents the mean ± SEM of 9 samples per participant. The results are adjusted for the effects of sampling method and repeated sampling, thus the error bars mainly represent intraindividual variability.

*Sampling method*

Adjusted for interindividual variability and allergy status, staining indices for CD45+ cells did not differ significantly between the spray blow technique (58 ± 1.6%) and the nasal lavage technique (53.5 ± 1.6%, p=0.8), but were significantly lower in swabs (48 ± 2%, $p < 0.01$, Figure 2). Relative neutrophil counts did not differ between nasal blow- and lavage technique (p=0.2), but with the swab technique, lower relative counts were obtained ($p < 0.05$). Like CD45+ cells, blow and lavage samples were not influenced by repeated sampling (p=0.4), but swabs were ($p < 0.05$). If adjusted for the allergy status, the staining indices for EPO+ cells were not influenced by the sampling method (p=0.4).

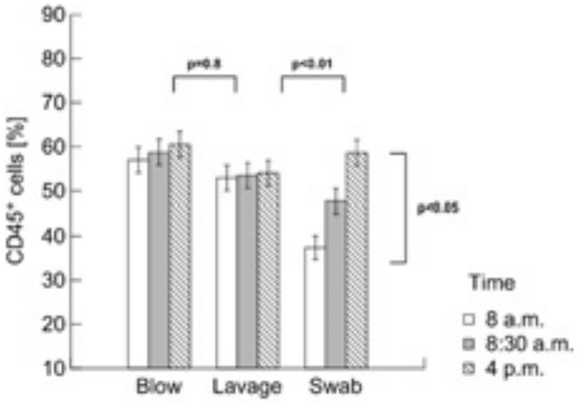


Figure 2. Influence of 3 sampling techniques and repeated sampling on the relative number of CD45+ cells. Sampling with swab yielded to the same relative number of CD45+ cells than with nasal lavage (p=0.8, n=16) but was significantly lower in swabs ($p < 0.01$, n=16). Repeated sampling did not significantly influence the results of nasal spray-blows or nasal lavages (p=0.8, n=16), but interfered with swab ($p < 0.05$, n=16).

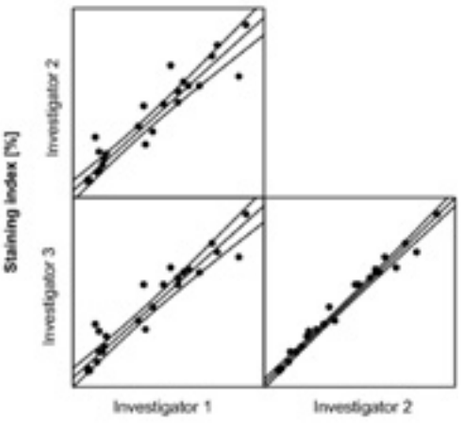


Figure 3. Scatterplots with staining indices of immunostained cells in nasal cytologies as judged by 3 blinded investigators (regression line with 95% confidence bands). A high interobserver concordance (Cohens $\kappa_w$ 0.95, $p << 0.001$) was observed.

*Repetitive sampling*

For CD45+ cells repeated sampling did not significantly influence the results of nasal spray-blows or lavages ($p > 0.5$), but interfered with nasal swabs ($p < 0.05$, Figure 2). Also a trend toward higher values with repeated samplings was observable for EPO+ cells, but this effect was not significant (p=0.17).

*Allergic status*

Participants with nasal allergy had less CD45+ cells (42 ± 2%) in their nasal cytologies than their non-allergic counterparts (57 ± 1%, p=0.02). Irrespective of the sampling method, subjects with nasal allergy had considerably less neutrophils (21 ± 3%) in their nasal cytologies than non-allergic participants (40.5 ± 1.6%, p=0.02). EPO+ cells were more frequent in allergic participants (22 ± 1%) than in non-allergic participants (8.5 ± 0.5%, $p < 0.001$).

*Interobserver variability*

To asses interobserver variability, 32 randomly chosen immunostained samples were evaluated by three blinded observers. The Cohen's weighted Kappa-coefficient served as parameter of concordance between two observers. A high concordance of 0.96 ($p << 0{,}001$) between observer 1 and observer 2, of 0.98 ($p << 0{,}001$) between observer 2 and observer 3 and 0.95 ($p << 0{,}001$) between observer 1 and observer 3 was found (Figure 3).

## DISCUSSION

Several techniques for sampling and evaluation of nasal cytologies are described [17]. In this study, nasal lavage, a nasal spray blow technique and absorption of nasal secretions to standardized polyurethane foam samplers (swab) were used in parallel for recovering cells from the nose of healthy and allergic volunteers within the same technique conducted at each subject three times in one day (08:00, 08:30 and 16:00 h). Subjects were students of the University of Ulm not representing the total population concerning age and social status. A total of 16 x 4 x 3 specimens was collected. Each specimen was stained with the May-Grünwald-Giemsa method and immunocytochemically with antibodies specific for almost all leukocytes (CD45) and specific for eosinophils (EPO). The staining index was given for both staining methods as percentage of positive cells to all counted cells.

*Sampling method*

The results of quantitative cytology in nasal secretions indicate a high interindividual variability already at healthy subjects. Near by, the reasons for this variability are multifaceted in which sampling has a considerable impact on it. Nasal lavage is a widely spread and the most commonly used sampling technique for nasal cytologies. It is easy to perform, but nevertheless there is no international standardization described for this technique. Lavage and spray-blow techniques combine several advantages like tolerability and learnability by all volunteers and patients, minimal trauma, minimal disturbance of the underlying physiological or pathophysiological process and particularly repeatability [3]. The lavage fluid reaches most areas of nasal mucosa exposed to the respiratory airstream as well as the main nasal mucus transport pathways originating in the paranasal sinus system. It is thus assumed that nasal lavage fluid gets in contact with most mucosal areas relevant in nasal physiopathology [18]. They are non-traumatic, easily manageable, and reproducible amounts of sampling fluid and cells are recovered [19].

An alternative technique to collect nasal cytologies employs a sampler with absorptive properties placed within the nasal cavity. Cellulose sheets, cotton tampons, paper discs and stripes, and polyurethane foam samplers have been used for this purpose. These techniques are suspected to cause trauma to the nasal mucosa and consequently alter the cellular composition of nasal smears, particularly if samples are obtained repeatedly. Sampling with swabs resembled a superficial curettage whereas cells were actively discarded from the surface of the nasal mucosa. Employing a swab technique, a significantly lower proportion of obtained cells were CD45+ than with nasal lavage or spray blow techniques at the first sampling time (Figure 2). It is likely that superficial epithelial cells are preferentially obtained by this technique. Swab techniques thus appear particularly appropriate, when the function of nasal epithelial cells is to be assessed, e.g. in ciliary function tests or for examinations of dysplasia for example after occupational exposure to wood dust. The nasal lavage and the nasal spray-blow technique are appropriate for the examination of nasal inflammation. Easy handling in field studies, minor exposure for subjects and adequate amounts of regained secretions are the main advantages of the nasal spray-blow technique. Both methods, the nasal lavage and the nasal spray-blow technique, are sparing and also convenient for repetitive sampling.

*Repetitive sampling*

Occasionally a study design requires repeated cell-sampling, for example before and after a challenge with an agent under investigation or before and after a work shift. The effect of repeated sampling on the results may be serious. It was not examined so far to what extent the parameters sampling technique, subjective influence of the observer and repeated sampling affect the results of quantitative nasal cytology. To determine the effects of repeated sampling on quantitative cytology of nasal secretions all specimens were evaluated visually by one observer using the laboratory internal SOP. Shifting of nasal biomarkers after repeated sampling is a common problem [9]. In this study a time dependent increase of positive cells was found for CD45 and EPO for the three sampling techniques. This may be due to the preceding sampling steps that evoke an inflammatory stimulus in the nasal mucosa. Particularly when sampling was performed with nasal swabs there was a marked increase of staining indices by the last sampling step. Unlike nasal swabs, for nasal lavage the increase of staining indices after repeated sampling was much lower. The nasal lavage is in contrast to the nasal swabs a sparing and non-traumatic technique to obtain cells from the nasal mucosa. Probably this is the reason for the lower staining indices after sampling with the nasal lavage.

*Allergic status*

Further on, there is no data about the interaction between these parameters and the individual allergic status of the involved subjects. It is ambiguous why allergic subjects had comparatively high staining indices when cells were collected with the sparing nasal lavage. Independently of sampling method and sample repetition, allergic subjects had lower relative counts for neutrophils ($p=0.08$) in the conventional May-Grünwald-Giemsa staining and lower staining indices concerning CD45 positive cells ($p=0.07$) compared to none allergic subjects. For patients with hypereosinophilia no more leukocytes and a decrease in the number of neutrophils (whereas the number of eosinophils increased) were found compared with the healthy control group [20].

*Interobserver variability*

Among sampling, it is the subjective influence of the observer that may bias the results of quantitatively analyzed cellular elements in nasal secretions. Other than in blood smears, there are cell elements, debris, dust, bacterial contaminations and highly viscous mucus strands in nasal smears [21]. The heterogeneity of nasal smears results in an intensive background staining, especially in immunocytochemically stained specimens. This aggravates quantification of positively stained cell elements. First of all, the subjective influence of three independent observers on the results of quantitative cytology was evaluated. On the basis of a laboratory internal standard operating procedure (SOP) an experienced biologist, an experienced assistant medical technician and an unskilled student evaluated 32 immunocytochemically stained specimens. Those were randomly chosen from a pool of 288 immunocytochemically stained specimens since evaluation of all specimens would have been an extraordinary effort. The results of quantitative cytology showed a very well compliance between the three observers. Cohen's "weighted kappa"-coefficient between observer 1 and observer 2 was 0.96 ($p << 0{,}01$), 0.98 ($p << 0{,}01$) between observer 2 and observer 3 and 0.95 ($p << 0{,}01$) between observer 1 and observer 3. Compliance was best between observer 2 and 3. A reason for this could be that observer 3 was introduced in the evaluation of quantitative cytology in nasal secretions by observer 2. However, the use of a laboratory internal SOP yielded to a uniformity of compliances. This indicates that the variability of staining indices is only in a minor amount caused by the subjective influence of different observers. In fact immunocytochemically stained cells are identified and afterwards classified in either positive or negative cells.

In conclusion, the present study showed that the interobserver variability could be minimized using a laboratory internal SOP. We would prefer the nasal lavage for further investigations on nasal cytologies, especially when repeated sampling is required.

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