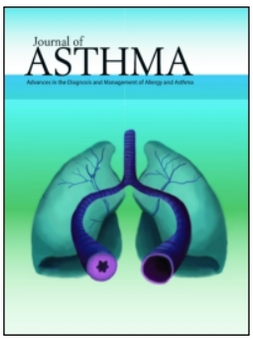

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Processing small sputum samples: a method validation study

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ABSTRACT

Background: Eosinophilia is frequently a feature of asthma. Sputum analysis can help with the diagnosis and phenotyping of asthma. The current gold standard method is unsuitable for samples <100 mg. However, children frequently produce samples below this threshold. *Aim:* To compare and validate our modified, small sample (>10 mg and <100 mg) sputum processing method (which omits sample filtering), with the current gold standard. *Method:* Prospective study of 32 adults with severe asthma providing sputum samples of sufficient size for dual processing. *Results:* The median (IQR) sample weight was 211.0 (162.4–185.5) mg and 57.5 (22.0–61.6) mg for standard, and small sputum sample processing respectively. There was no statistically significant difference in the median (IQR) cell counts between Method A and B, respectively: eosinophils 3.8% (1.5–14.0) versus 4.9% (1.3–15.5); neutrophils 78.1% (46.5–92.4) versus 65.0% (48.3–86.6). *Conclusion:* The small sputum sample processing is feasible and reliable, and yields similar results to standard processing.

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KEYWORDS
asthma; child; diagnostics; eosinophils; pediatrics; phenotypes

Background

Eosinophilic airway inflammation is often a feature of asthma [1]. Sputum analysis can help with the diagnosis and phenotyping of patients with asthma, and to predict corticosteroid responsive disease. The monitoring of sputum eosinophils with titration of corticosteroid dose has been shown to reduce asthma exacerbations in adults [2]. Sputum induction for inflammatory cell analysis is relatively noninvasive and provides a repeatable measure of airway inflammation.

The current gold standard sputum processing method [3] is not suitable for samples <100 mg. Children, however, frequently produce only small samples below this threshold. In response to this common problem, we developed a modified sputum processing method suitable for smaller samples, >10 mg but <100 mg. The key difference between the small sample method and the gold standard is the omission and replacement of the sputum filtration step with a micro-centrifugation step.

Our study aim was to compare and validate our modified, small sample, sputum processing method (Method B) with the current gold standard (Method A).

Method

Thirty-two adult patients attending the difficult asthma clinic provided sputum samples of sufficient quantity for dual processing between August 2013 and January 2015. This study obtained ethics approval with the Research Ethics Committee reference number 08/H0406/189.

Sputum collection: Sputum samples were generated either spontaneously or by induction using nebulized hypertonic saline [3]. Samples were sealed, placed on ice and processed within two hours.

Sputum selection: Under sterile conditions, small (<100 mg) and large (>100 mg) aliquots of sputum were selected from each sample.

Method A: Processing using the gold standard has been previously described in detail [3].

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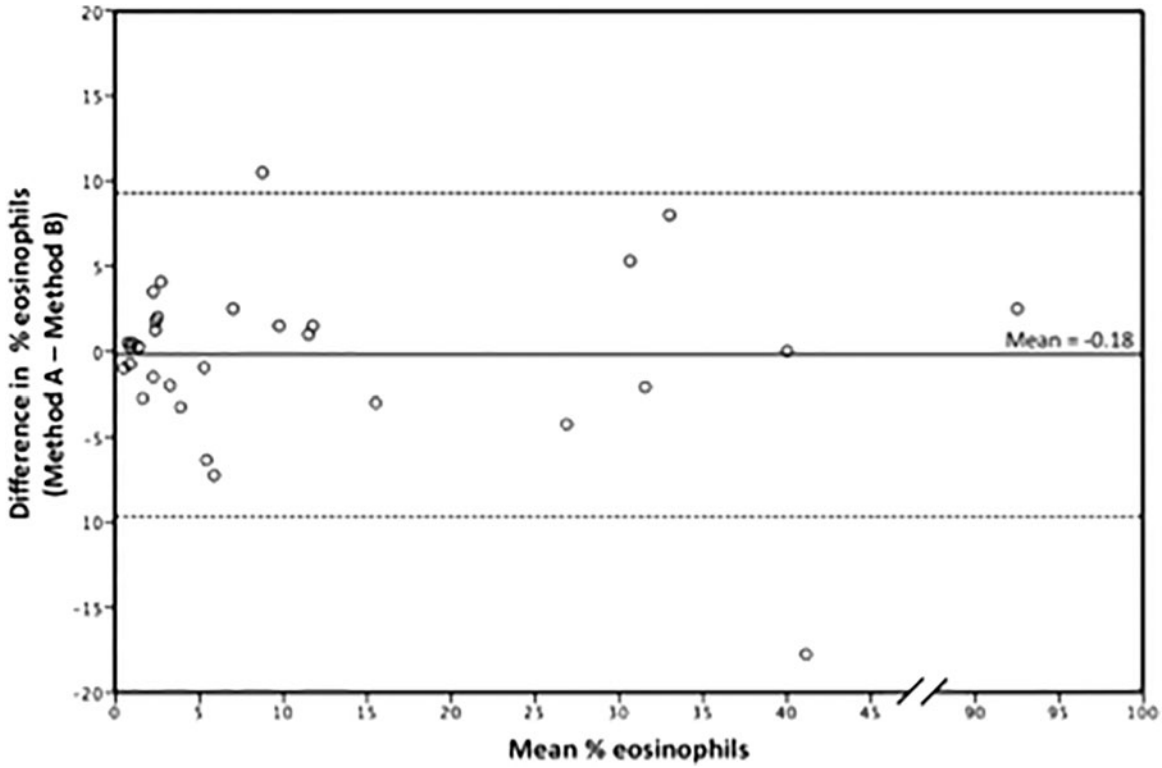

Figure 1. Bland-Altman plot of sputum eosinophils percentages determined by Method A versus Method B Comparison of the eosinophil percentage as determined by Method A (small sample) versus Method B (gold standard) with the difference in eosinophil percentage plotted against the mean of both values. Solid line: mean of differences; dotted lines: 95% limits of agreement, calculated from the mean difference +1.96 standard deviation of the difference.

The main difference between the gold standard and small sputum sample methods is the replacement of the filtration step with micro-centrifugation in the latter.

Method B: The smaller sample was micro-centrifuged (Micromax, IEC, Needham Heights, MA) for 4 s at 3000 rpm. This is sufficient to concentrate larger debris in the axis of the tube without causing complete sedimentation. The sample was macroscopically inspected and any contaminating material removed using a micropipette, before reweighing.

Cell counts and slide preparation: Total cell count (TCC), viability and level of squamous cell contamination was calculated for each sample using a Neubauer hemocytometer and the Trypan blue (Sigma, Poole, UK) exclusion method. Cytospin slides were generated using our standard protocol [3] and differential cell counts (DCC) of at least 400 non-squamous cells were performed under a microscope by an experienced single investigator (WM) blinded to the origin of each slide.

Data handling: Statistical analysis was performed using SPSS Statistics version 22.0.0.1 (SPSS Inc., Chicago, IL). Data were expressed as mean and standard deviation (SD), or median and interquartile range (IQR) as appropriate and compared using paired sample t-tests and Wilcoxon signed-rank tests respectively. Method comparison was performed as advocated by Bland and Altman [4] with the difference in cell counts (Method A – Method B) plotted against the mean (Method A + Method B)/2, given with 95% limits of agreement. Systematic bias was assessed by inspection of scatter plots and linear regression analysis.

Results

Sputum samples from 32 patients (19 female, 13 male) and median age of 58 years (range: 19–84 years), with severe asthma were split and processed simultaneously by both methods. The median (IQR) sample size used for Method A was 211.0 (162.4–185.5) mg and for Method B was 57.5 (22.0–61.6) mg. The median (IQR) total cell count was 0.65 $(0.44 - 1.18) \times 10^3/\mu$L.

There was no statistically significant difference in the median (IQR) cell counts between Method A and B, respectively: eosinophils 3.8% (1.5–14.0) versus 4.9% (1.3–15.5), $Z = -0.157$, $p = 0.875$; neutrophils 78.1% (46.5–92.4) versus 65.0% (48.3–86.6), $Z = -1.903$, $p = 0.057$; squamous cell contamination 2.7% (0.0–7.7)

versus 0.7% (0.0–6.3), $Z = -1.412$, $p = 0.158$. Cell viability was slightly lower in samples processed by Method B 60.0% (29.4–78.5) compared to Method A 69.9% (47.1–89.0), $Z = -3.449$, $p = 0.001$.

Agreement in eosinophil percentage between methods is shown in a Bland-Altman plot (Figure 1).

The mean difference in sputum eosinophils between the two methods was −0.18%; precision is illustrated by the 95% limits of agreement. There is no evidence of systematic error or proportional bias (linear regression analysis of mean difference against mean yields $p = 0.776$). In total, 19 (59%) patients demonstrated sputum eosinophilia ≥3% by Method A and 20 (63%) by Method B.

Lin's concordance correlation co-efficient (rc) was performed for the 25 patients with eosinophils >3% through method A or B. This resulted in an rc of 0.965 (95% CI 0.922–0.984).

For neutrophils, the mean difference between methods was 5.99%, 95% limits of agreement from −33.6 to 45.8%. Again, observation of Bland-Altman plot (not shown) showed no evidence of systematic error or proportional bias (linear regression $p = 0.813$).

Discussion

This study demonstrates that an alternative method of sputum processing suitable for small samples is feasible and reliable. We obtained a leukocyte differential cell count using both methods from all samples included in this study. When validating the novel method using micro-centrifugation against the gold standard technique employing gauze filtration, we found that the proportion of eosinophils and neutrophils in sputum are comparable between the two methods.

We found a mean difference of 0.2% in the eosinophil percentage between the two methods, which is unlikely to be of clinical significance.

For the gold standard method, filtration of the sputum is performed to reduce salivary and squamous cell contamination and to remove debris in order to ensure high quality cytospin preparation. Filtration reduces TCC [5] making it impracticable to analyze small samples. Occasionally this is a problem in adults, and this is a common problem in the pediatric population, particularly young children.

Our modified processing protocol results in good quality cytospin slides and retains a sufficient number of cells to perform sputum DCC, despite the smaller sample size. The very low squamous cell contamination and high cell viability are indicators of the good sample quality using either method. Moreover the alternative sample processing method is no more time-consuming and allows the processing of multiple sputum samples simultaneously. Also, only standard laboratory equipment is required.

The findings from this study in patients with asthma are applicable to the investigation of sputum obtained from patients with other respiratory conditions, such as bronchiectasis.

Limitations: We did not investigate intra-method repeatability of our modified method. The focus of this study was on agreement between methods and not on clinical characteristics of patients, many of whom were giving routine pre-clinic samples. Due to issues relating to sample quantity, the method comparison study only included sputum from adult patients with asthma.

In our study, the operator macroscopically inspected the sample, eliminating the contaminating material with a micropipette. We could have opted for other methods to eliminate unprocessed mucous and cell debris in small samples such as small cell strainers with or without the centrifugation step, which would have been less operator-dependent and more easily standardized.

Conclusion

We tested an alternative sputum processing method, suitable for small samples <100 mg, to estimate the leucocyte differential cell count. We found this method performed well against the current gold standard. This novel method will increase the number of viable sputum samples obtained in practice and facilitate the wider adoption of sputum testing in patients, especially in children, with asthma and other relevant respiratory conditions.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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