

## CORRESPONDENCE

# Validation of assays for inflammatory mediators in sputum

To the Editor:

We read with interest the article by STOCKLEY *et al.* [1] in which an enzyme assay for myeloperoxidase (MPO), and immune assays for MPO, interleukin (IL)-8, leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and secretory leukoprotease inhibitor (SLPI) were validated in sputum from patients with chronic lung disease. We commend them for performing an important and often overlooked task of validating assays in sputum, but we are unclear on several points.

Elastase levels were measured in eight pooled mucoid and eight pooled mucopurulent sputa. No elastase was detected in the pooled mucoid sputa and therefore, it was assumed that these sputa contained very little elastase. However, some individual mucoid sputa may have had high levels and some mucopurulent sputa low levels. These differences would not be evident in the pooled sputum, and the assumptions may not be true. The reason for pooling the sputum was not explained by the authors in the methods or discussion.

Spiking (addition of a known amount of pure mediator) was performed just before the assay; it would be more relevant to have spiked the sample before processing the sputum. In calculating the recovery of the spiked mediator, the amount measured by assay of the spiked sample was subtracted from the expected level. However, subtraction of the level of endogenous mediator from the spiked sample would have given a more accurate calculation of the recovery of the spiked amount. Instead, depending on the concentration of endogenous mediator present, the recovery is likely to have been overestimated.

The volume of standard added to the wells in the microtitre plate of the MPO enzyme assay was stated to be 10 mL. Was this an error since a more realistic volume would be 100 µL? The results of the spiking with MPO showed that there was poor recovery with the immune assay compared to the enzyme assay. It would be interesting to know if there was any correlation between endogenous levels of MPO measured by each method, and if the immune assay, although resulting in a lower spiked recovery, was able to detect higher levels of MPO than the enzyme assay. The numbers in these assays were small, n=6, in the IL-8, SLPI and MPO assays, n=4 for the LTB<sub>4</sub> assay. Was the study sufficiently powered to detect differences between the levels with these small sample sizes? The authors do not state if the samples assayed were mucoid or mucopurulent, which would be important to know, since they stress the potential effect of proteases on the assays.

Dilution of the sample before immune assay resulted in greatly increased levels of myeloperoxidase, suggesting the presence of interfering substances. Such interfering substances may mask the myeloperoxidase epitopes, so that they are not recognized by the assay antibodies, or it could affect the assay antibodies directly. Binding proteins, such as α<sub>2</sub>-macroglobulin [2] can potentially mask epitopes, and proteases could damage the assay antibodies. The authors do not speculate on the nature of this interfering substance except to suggest that it is elastase. It would have been interesting to correlate elastase levels in individual sputa with the increase in levels with dilution. If an elastase inhibitor had been used, this would

have helped discriminate between the effect of elastase on the assay or a masking effect on myeloperoxidase epitopes.

**M. Kelly, R. Leigh, F.E. Hargreave**

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## References

1. Stockley RA, Bayley DL. Validation of assays for inflammatory mediators in sputum. *Eur Respir J* 2000; 15: 778–781.
2. Feige J-J, Negoescu A, Keramidis M, Souchelnitskiy S, Chambaz EM. α<sub>2</sub>-Macroglobulin: a binding protein for transforming growth factor-β and various cytokines. *Horm Res* 1996; 45: 227–232.

From the authors

We have reviewed the letter from M. Kelly, R. Leigh and F.E. Hargreave. We thank them for the letter and in particular their comments on the importance of validating assays in sputum as this was the primary purpose of our article. However, they have raised several other issues that are of importance to address.

The authors are correct that we pooled a variety of samples of similar characteristics, and the purpose was to enable us to have sufficient volume to be able to carry out all the experiments in the same medium, as usage of different samples for different parts of the experiment would have been severely open to criticism. Elastase activity in the pooled mucoid sputum was negative. Neutrophil elastase comes from the neutrophil and the number of neutrophils present in the secretions reflects the purulent nature. In mucoid samples although neutrophils are present the numbers are low. We have repeatedly shown in our 20-yr experience that it is very

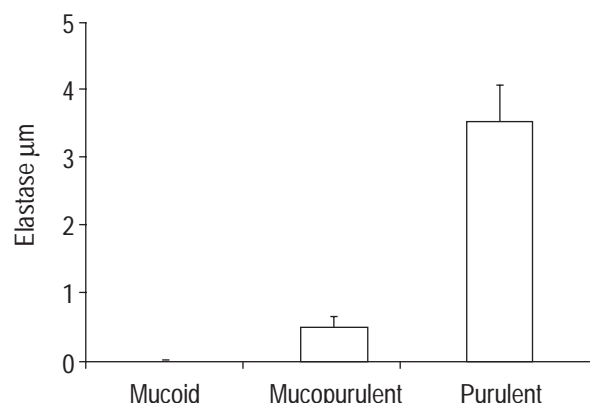


Fig. 1. – Histograms represent mean ± SEM bar of elastase activity for sputum samples separated by their macroscopic appearance. Free elastase was not detected (<0.63 nM) in 14 mucoid samples and 9 of 31 mucopurulent samples but all (n=18) purulent samples.

rare to find neutrophil elastase activity in these samples, (fig. 1) although of course a small amount of immunologically detectable neutrophil elastase is present in some. This immunological elastase is nearly always inhibited by the presence of proteinase inhibitors and in particular secretory leukoprotease inhibitor (SLPI) and  $\alpha_1$ -antitrypsin.

In mucopurulent sputum samples elastase activity is more commonly detectable and is present in most samples. This will, however, relate to the number of neutrophils because there is a sufficient need for them to be present to release enough elastase to overcome the local inhibitors. These samples always contain detectable elastase by immunological methods and again the pool that we studied here had easily detectable elastase activity ( $\mu\text{M}$ ) we would agree that occasionally this activity may be absent but again this was not the purpose of the publication. Whether elastase activity is present or absent is not of importance unless the elastase activity is responsible for the loss of the mediator signal and, or course, in the validation assays that are carried out this should always be checked. In the assays reported in our paper the evidence would suggest that elastase activity does not interfere with the assays except possibly the myeloperoxidase (MPO) immune assay and SLPI.

The point about spiking the eventual sample that we assay rather than the total sample was merely to make a point about assay validation. It is quite true that the total recovery can also be affected by the way the sample is processed. For instance, dithiothreitol (DTT) breaks down the mucus portion of sputum and although DTT itself can interfere with some assays it will also release cytokines and other proteins that are bound to the mucopolysaccharides. Again, these are studies that we are currently performing although as a rule we do not treat our samples with DTT. We are more interested in the sol phase because this is the freely diffusible component of the secretion and it is a portion of the sample that we have studied over 20 years having previously validated the processing method.

The comment about the typographical error is absolutely correct and this should read 10  $\mu\text{L}$  not 10 mL.

We have looked at the method for assessing recovery and have read both the comments by Kelly *et al.* as well as our own paper. I think we mean the same thing, in other words: we assayed the sample once; added a known amount; obtained the final result and hence, determined how much it had gone up by. This was then subtracted from the predicted amount that it should have gone up by to obtain the per cent recovery. On reviewing our wording we still feel that this explained what we did, although, having the issue raised by the reader we can understand how a failure of clarification has emerge. Nevertheless, by recovery we mean not the proportion of the total signal *i.e.* endogenous plus spike, but the recovery of the spike proportion of the signal.

We have found some difficulty in determining what the authors are alluding to here. If they are asking does  $n=6$  enable us to say that the decrease that we have got with the spike is significant the answer is no, but that was not the point. From

our point-of-view recovery is fairly good and therefore, acceptable within the limits stated in the publication. If on the other hand they are concerned whether  $n=4$  for the MPO immune assay means that the differences we find with dilution are significant the answer is clearly yes. If the standard deviation and mean values are taken into account its very clear that there is no overlap of data and  $p<0.01$  for all the differences. Again, it was not really the purpose of the publication to state how significantly different any overestimation with this immune assay was.

All coefficients of variation (CV) and recovery studies were carried out in both the mucoid and mucopurulent pool. Again this was a point that we did not belabour but in the paragraph on assay validation we indicated that we assayed both the mucoid and mucopurulent sol phase. This was mentioned again in the subsequent paragraph of this section where we commented on the sample dilution and it was clearly expanded upon with the myeloperoxidase immune assays. Suffice to say that all the data presented was the same, whether the results are obtained with mucoid or mucopurulent samples.

Finally, it should be emphasized when referring to the figures that the CV's are not the data on the figure or the sample numbers being shown on that figure, they are the data obtained from replicate assay of the individual samples and are included as a separate addition to the figure captions. Again, we thought that this was clear in our original article.

The final point about factors that interfere with the assay I would point out that we have suggested both interference and elastase could effect the results. Indeed, we demonstrated that elastase does affect the results for myeloperoxidase but also that mucoid samples influence the results when elastase is not detectable suggesting that elastase is not the only factor that could influence the results.

The remaining comments about inactivating elastase or comparing the elastase content with the under or over-estimation of MPO would be interesting to carry out and we look forward to the results being generated. However, from our point-of-view this is not an assay we would wish to use since it is not reproducible even within mucoid samples where elastase activity is not present. Again, the effect of  $\alpha_2$ -macroglobulin is of interest, although, I am not aware that it affects MPO. Certainly, there is more  $\alpha_2$ -macroglobulin in mucopurulent samples and very little in mucoid samples therefore, again it is unlikely to be responsible for the major problems seen within these samples.

Finally in summary it is not, nor has it been, our purpose to determine the reasons for poor assay recovery but merely to confirm that assays work in samples that we assess. We feel the important message is that others should confirm this in their own samples with their own methods.

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