Determining the relationship between neutrophil elastase and salivary α-amylase in cystic fibrosis sputum and oral samples

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Abstract

Cystic fibrosis (mucoviscidosis) is an autosomal recessive genetic disorder with a poor prognosis and no known cure. Caused by mutations in the gene for cystic fibrosis transmembrane conductance regulator (CFTR), a protein that regulates the viscosity of bodily fluids, cystic fibrosis leads to the buildup of viscid mucus in the lungs that provides pathogens an optimal environment to thrive. As a result, CF patients face frequent infections and must regularly use ineffective general antibiotics. Therefore, microbiome sequencing of CF patients is critical to the development of targeted treatments. However, as samples are taken orally, saliva contamination is an issue and may cause samples to misrepresent sequencing results. To determine the severity of contamination, we conducted assays on sputum and mouth rinse samples of CF patients and calculated the concentrations of two specific enzymes, neutrophil elastase (NE) and salivary α-amylase (SA), that are found mainly in sputum and oral samples, respectively. Neutrophil elastase, a serine proteinase that destroys pathogens during infection, is known to be found in high levels in CF patients, while salivary α-amylase is equally present in both the CF and healthy population. By comparing concentrations of neutrophil elastase and salivary α-amylase, we can calculate the level of contamination for each sputum and oral sample. Levels of contamination can be used for future reference in regards to sample procuring procedures, while samples that show less contamination can be sequenced to identify the strains of bacteria present during a cystic fibrosis infection.

Methods

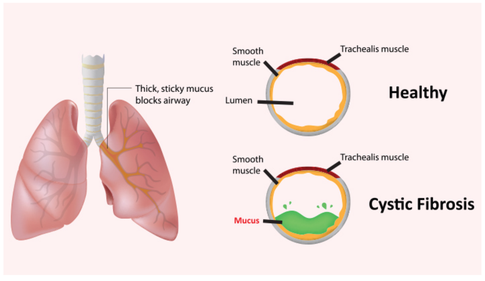

Fig 1. Cystic fibrosis causes high viscosity in mucus, creating a prime environment for infection.

For optimal NE assays, sputum samples were diluted by a factor of 200, while mouth rinse samples were diluted by 40. 0.3 mg/mL NE concentrate was diluted by a factor of 200 and used as the positive control. For SA assays, sputum samples were diluted by a factor of 4000, and mouth rinse samples were diluted by a factor of 200. SA concentrate at 1.0 mg/mL was then diluted by a factor of 200 and used as the positive control.

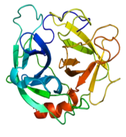

Fig 2a. Neutrophil Elastase

Fig 2b. Salivary Amylase

For both assays, a 1/1000 dilution of tween-20 (polyoxyethylene sorbitan monolaurate) into phosphate-buffered saline was used as assay buffer and thus, the negative control.
In all wells, specific fluorogenic substrates were prepared as targets for the enzymes being tested. With the cleavage of the substrates by the enzymes, emitted fluorescence from the substrate was calculated by the assay reader at a specific excitation/emission wavelength in order to determine the relative amounts of enzymes. In the NE assays, the Suc-LLVY-AMC peptide was used, while in SA assays, a solution of starch-based conjugate and solvent was used.

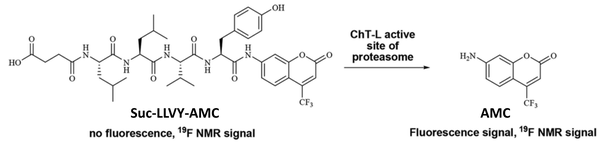

Fig 2c. NE Substrate Fluorescence

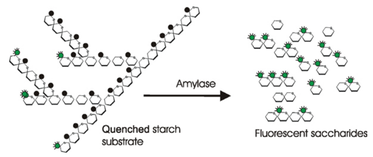

Fig 2d. SA Substrate Fluorescence Process

25 µL of each diluted sample were pipetted into 25 µL of specific substrate in triplicate wells to mitigate error. Assays were then analyzed by the assay reader for amount of RFU after 150 seconds of incubation. The data output was then organized, averaged, and analyzed for possible correlations.

Fig 2e. 96-well assay plate

Results for NE/SA Assays

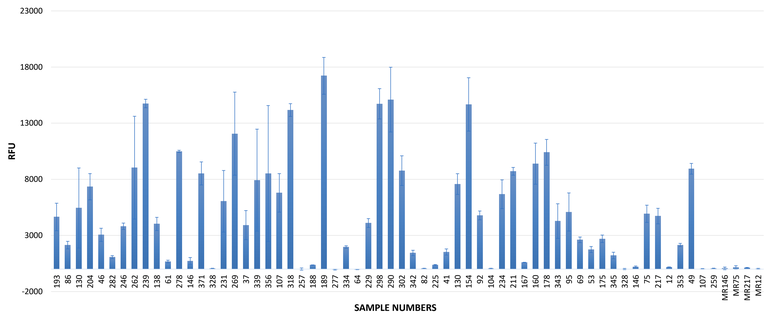

Fig 3a. NE Fluorescence Results
λ = 360/460 nm

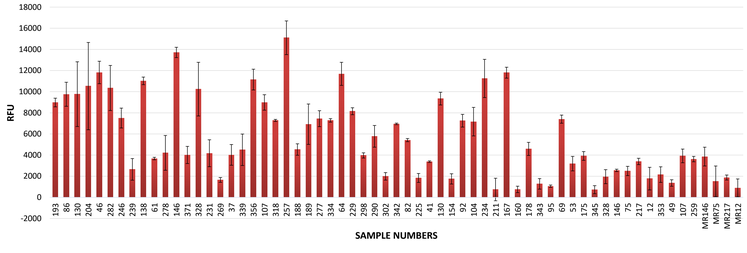

Fig 3b. SA Fluorescence Results
λ = 485/528 nm

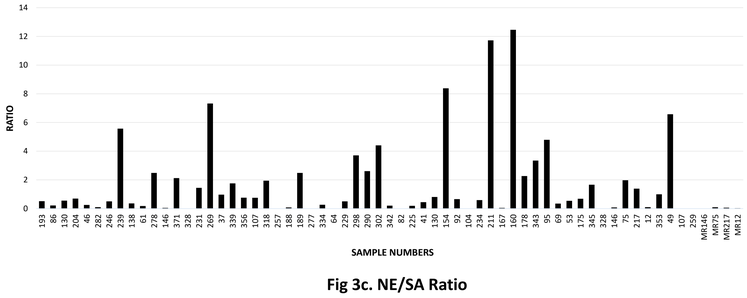

Fig 3c. NE/SA Ratio

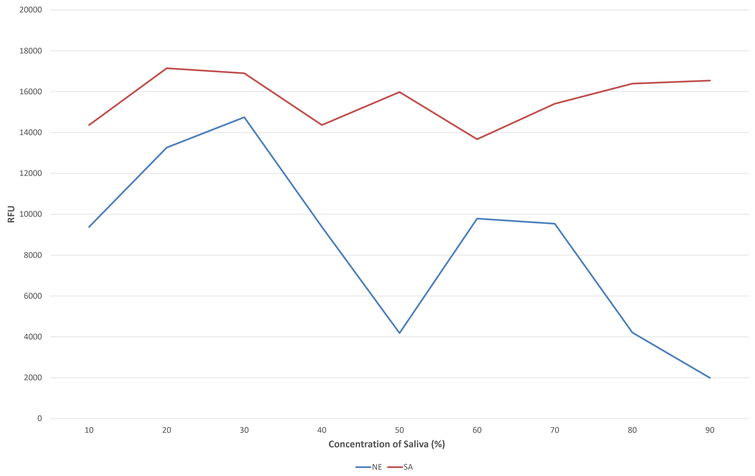

Fig 3d. Blind Samples Fluorescence Results

Analysis and Conclusions

It can be seen that sputum samples generally show a strong presence of neutrophil elastase that is significantly larger than the positive control. In addition, mouth rinse that was tested for NE gave weak signals of the enzyme's presence as expected. Thus, we conclude that levels of NE differ in CF patients due to the varying levels or outright absence of inflammation and infection at the time of testing. In addition, sputum samples had a large range of salivary α-amylase presence. We conclude that this variation may be attributed to the unpredictability of sputum procurement; that is, patients produce a variable amount of saliva when dispensing sputum. However, sputum on average had higher levels of SA than mouth rinses, possibly because the mouth produces more saliva when making the effort to cough. Negative controls in both assays showed little enzyme activity as expected. Furthermore, the inclusion of blinds in the experiment provided an additional control group; the resulting data shows a relatively stable amount of SA and decreasing amounts of NE as the saliva concentrations increases. We attribute this to the fact that sputum may contain SA that compensates for changes in saliva concentration.

The difficulty of obtaining uncontaminated sputum samples is problematic, and all samples are inevitably contaminated with saliva and saliva-residing bacteria. We conclude that SA contamination originates from the unpredictable sample procurement process and argue that contamination may be reduced through changes in the procedure. Nevertheless, we are hopeful that our work can facilitate the successful sequencing of the CF microbiome and the quest for a cure for this chronic ailment.

References

Quinn RA, Lim YW, Mak TD, Whiteson K, Furlan M, Conrad D, Rohwer F, Dorrestein P. (2016) Metabolomics of pulmonary exacerbations reveals the personalized nature of cystic fibrosis disease. PeerJ 4:e2174.

Liu H, Lazarus S, Caughey G, Fahy J. (1999) Neutrophil elastase and elastase-rich cystic fibrosis sputum degranulate human eosinophils in vitro. American Journal of Physiology 276:L28-34.

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