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Adaptive Focused Acoustics Processing to Aid Recovery of Fungi from CF Patient Sputum Samples

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Abstract

Background: Cystic fibrosis (CF) is a genetic disorder that afflicts over 70,000 people worldwide. It is characterized by viscous mucus that accumulates in the lungs creating challenges for microbiology labs processing sputa to isolate slower growing fungal species. Our laboratory attempted to liquefy and homogenize mucoid sputum samples by exposure to Adaptive Focused Acoustics™ (AFA) in order to increase recovery of fungal pathogens.

Methods: Twenty sputum samples were processing by AFA using Covaris S220x Focused ultrasonicator (Covaris Inc., Woburn, MA). Variables considered included power input, time of exposure, sample volume and mechanical disruption. Once optimized, four fungi commonly isolated from CF patients (*Trichosporon mycotoxinivorans*, *Scedosporium apiospermum*, *Exophiala dermatitidis*, *Aspergillus fumigatus*) were selected to determine AFA's potential effect on viability. They were first processed in sterile saline with subsequent emulsions plated in parallel with unprocessed stock. Next, non-CF patient mucoid sputum was pooled and spiked with fungal isolates above; samples were diluted 1:1 with sterile saline. CF sputum was diluted but not spiked. Prior to AFA, samples were inoculated to blood agar plates (BAP) and Sabouraud Dextrose (SAB) plates. Samples were processed by AFA until homogenized and liquefied, then plated to BAP and SAB. BAP incubated for 72 hrs at 37^{0}C and were read at 24 / 72 hrs; SAB plates were incubated at 25^{0}C and read over 14 days.

Results: Dilution (1:1) and AFA was successful in the homogenization and liquefaction of mucoid sputa, including those from our CF clinic. Optimal conditions: 100 Peak-Incident-Power / 50 Duty Factor / 200 Cycles per Burst for 15 sec. Processing by AFA did not affect the viability of fungal organisms either in solution or spiked into sputa. No effects were observed on fungal viability pre-and-post-AFA in CF sputa. Two CF sputum samples exhibited biofilm production on direct- BAP but resulted in isolated colonies on post-AFA BAP.

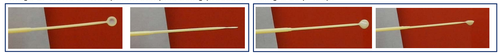
Conclusion: AFA paired with sample dilution offers an optimal process for the homogenization / liquefaction of viscous sputa while maintaining fungal pathogen viability. Additionally, it may disrupt biofilm formation, assisting in fungal recovery. Potential application of AFA to enhance fungal culture in CF patients warrants future studies.

Background

Cystic fibrosis (CF) is a genetic disorder that afflicts over 70,000 people worldwide.[1] It is characterized by viscous mucus that accumulates in the lungs creating challenges for microbiology labs processing sputa;[2,3] these challenges may contribute to an under appreciation of fungal diversity present in the CF lung.[3,4] Previous studies have used mucolytic agents[3,4,5] or direct sonication[5] to aid in the processing efforts of very mucoid sputa, but this comes with the risk of impact on viability and potential cross contamination.

Sputa samples seen in the lab vary widely in their consistency as illustrated by the collection of the two sputa samples from different patients, using a standard 10 µl loop (Image 1). Standardization of sample consistency could help with quantification of pathogens as well as recovery of sample.

Image 1: Views of two sputum samples being processed using a 10 µl loop.



Our laboratory attempted to liquefy and homogenize mucoid sputum samples by exposure to Adaptive Focused Acoustics™ (AFA) in order to increase recovery of fungal pathogens.

Methods

Phase I: Optimization

Twenty sputum samples were processed by AFA using a Covaris S220x focused ultrasonicator (Covaris Inc., Woburn, MA). We looked at variables such as power input, time of exposure, sample volume and mechanical disruption (fiber type). (Table 1) Endpoints were the observed homogenization and liquefaction of the samples. (Figures 1A-1D; Figures 2A – 2E)

Phase II-A: Fungal Viability

Four species of interest (*T. mycotoxinivorans*, *S. apiospermum*, *E. dermatitidis*, and *A. fumigatus*) were selected to test the effects of AFA on fungal viability. We ran the optimized protocol from Phase I, as well as 2X Peak Incidence Power (PIP), with both long and short fibers. Spikes of yeast cells or spores were created at ~10^{3} cfu/ml in sterile saline and processed on the Covaris S220x, subsequent emulsions were plated in parallel with unprocessed stock. In order to represent mold samples that would be seen in vivo, we created hyphae suspensions[6] of the *A. fumigatus* and *S. apiospermum* which were processed in parallel with the above. Plates were observed for up to 14 days and colony counts were performed. (Figures 3A-3C; Table 2A)

Phase II-B: Fungal Viability within Sputa

Non-CF patient mucoid sputum was pooled and spiked with fungal isolates above; samples were diluted 1:1 with sterile saline. Samples were processed by AFA using the optimized protocol from Phase I, until homogenized and liquefied. Both unprocessed and AFA-processed samples were inoculated to blood agar plates (BAP) and Sabouraud Dextrose (SAB) plates. BAP incubated for 72 hours at 37^{0}C and were read at 24 / 72 hours (data not shown); SAB plates were incubated at 23^{0}C (±2^{0}C) and read over 14 days. (Figure 3D, Table 2B)

Phase III: Modeling for future Studies

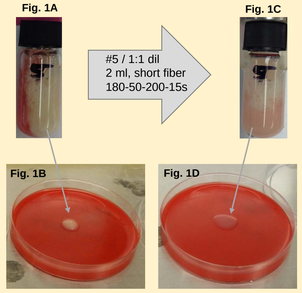
CF sputum samples were gathered and kept at 4^{o} until processing (within 5 days of collection). CF sputa was processed as Phase II-B samples above with no additional spike. Samples were plated and observed as above. (Figure 4A,4B) Results were compared to clinical laboratory records. (Table 3)

Results

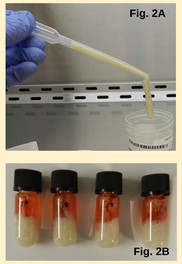
Phase I: Homogenization and Liquefaction

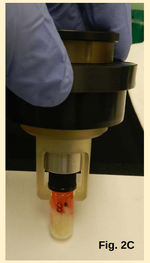
Table 1: Protocol Optimization

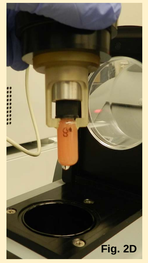
Sputa Pool	Tube	Dil	Fiber	Power	Cyc/Burst	Time to Change
1	1	1:1	short	200–50	200	15 sec
	2	1:1	long	200–50	200	15 sec
1a	3*	undil	short	200–50	200	15 sec
	4*	undil	long	200–50	200	15 sec
2	5	1:1	short	180–50	200	15 sec
	6	1:1	long	180–50	200	15 sec
3	7*	undil	short	150–50	200	15 sec
	8*	undil	long	150–50	200	15 sec
	9*	undil	short	100–50	200	30 sec
	10*	undil	long	100–50	200	30 sec
4	11	1:1	short	80–50	200	2 min
	12	1:1	long	80–50	200	30 sec
	13	1:1	short	50–50	200	1 min
	14	1:1	long	50–50	200	1.25 min
	15	1:1	short	100–50	200	15 sec
	16	1:1	long	100–50	200	15 sec
	17	1:1	short	150–50	200	10 sec
	18	1:1	long	150–50	200	< 10 sec
	19	undil	short	200–50	200	15 sec
	20	undil	long	200–50	200	15 sec

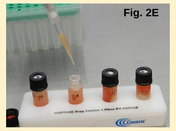


Figures 1A-1D: Sample processed with diluent
1A: Pre-AFA sample #5- 2 sputum samples of contrasting coloration/consistency pooled with sterile saline, **1B:** Pre-AFA sample plated to BAP **1C:** Post-AFA sample #5, **1D:** Post-AFA sample plated to BAP





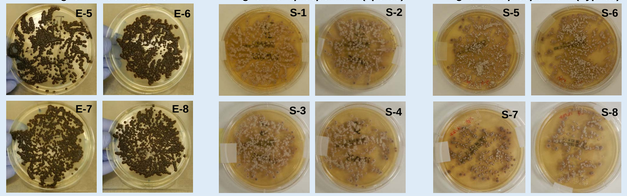




Figures 2A-2E: Sample processing
2A: Unprocessed patient sputum sample, transferred by sterile pipette with tip cut off. **2B**: Two viscous sputum samples of contrasting visual characteristics used to create similar pools. **2C**: Undiluted sputa pool pre-AFA **2D**: Undiluted sputa pool post-AFA, **2E**: Post-AFA sputum sample being transferred by standard 200 µl pipette.

Phase II-A: Viability of Target Organisms

Fig. 3A-C: Plated Saline Suspensions, Post-AFA



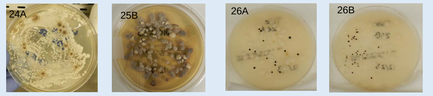
***E. dermatitidis* (E-5 – E-8)**, ***S. apiospermum* (spores [S-1 – S-4], hyphae [S-5 – S-8]),** *T. mycotoxinivorans* and *A. fumigatus* (spores, hyphae) (Not shown) were processed by AFA under the optimized setting from Phase I with the following modifications:
Tubes 1 & 5: 100PIP/50DF/200CycPerBurst/15sec, short fiber
Tubes 2 & 6: 200PIP/50DF/200CycPerBurst/15sec, short fiber
Tubes 3 & 7: 100PIP/50DF/200CycPerBurst/15sec, long fiber
Tubes 3 & 7: 100PIP/50DF/200CycPerBurst/15sec, long fiber

Table 2A: Recovery Data for Fungal suspensions

Fungi	Spike Conc. (cfu/ml)	Post−AFA Vol. Plated (ml)	Anticipated Colony Count	Actual Colony Count	Corresponding Images
E. dermatitidis	3270	0.100	327	>300	E−5, E−6, E−7, E−8
S. apiospermum (spores)	2880	0.100	288	>250	S−1, S−2 S−3, S−4
S. apiospermum (hyphae)	Quantification of hyphal elements was not possible; images shown to prove viability.				S−5, S−6 S−7, S−8

Phase II-B: Viability of Target Organisms in Sputa

Fig. 3D: Plated Spiked Sputum Samples, Post-AFA



***S. apiospermum* (spores [24A], hyphae [25B]), *E. dermatitidis* (26A,26B)**, *T. mycotoxinivorans* and *A. fumigatus* (spores, hyphae) (Not shown) were spiked into non-CF viscous sputum, processed by AFA:
A series: 100PIP/50DF/200CycPerBurst/15sec.
B Series: 200PIP/50DF/200CycPerBurst/15sec.

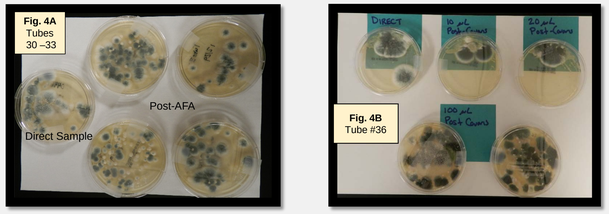
Table 2B: Plate Count Data for Spiked Sputum Samples

Fungal Spike	Spike Conc. (cfu/ml)	Spike Volume (ml)	Total Sample Volume (ml)	Post−AFA Plated (ml)	Anticipated Colony Count	Actual (Average) Colony Count
Exophiala	3270	0.100	2.0	0.100	16.35	23
S. apiospermum (spores)	2880	0.100	2.0	0.100	14.40	13
S. apiospermum (hyphae)	Quantification of hyphal elements was not possible; images shown to prove viability.					

Phase III: Application of Process on Sputa from CF Patients

Table 3: Clinical Lab Findings

Tube #	Clinical Lab Finding
28	Moderate Respiratory Flora
29	Light Respiratory Flora, Yeast
30	***A. fumigatus***, *S. maltophilia*, Achromobacter sp., *C. indologenes*
31	
32	
33	
34.1	*S. aureus*, *P. aeruginosa* (X4 morph.), Yeast, Achromobacter sp.
34.2	
35	Light Respiratory Flora
36	***A. fumigatus***, *P. aeruginosa*, *S. aureus*, Achromobacter sp.



Figures 4A, 4B: Examples of CF patient sputa microflora, post AFA processing
4A: Patient sputum sample was diluted 1:1 with sterile saline, processed in quadruplicate by AFA [100 PIP/50DF/200CycperBurst for 15 secs in a 2 ml Covaris tube with a short fiber. Sample grew out yeast and *A. fumigatus* on SAB plate at room temperature **4B:** Patient Sputum was processed as above. Aliquots were plated in volumes comparable to direct plating (10/20 µl) as well as 100 µl spread plateSamples grew out yeast and *A. fumigatus*.

Results Summary

Phase I: AFA is capable of consistent homogenization and liquefaction of mucoid sputa at 100 PIP/50DF/200 Cyc per Burst for 15 seconds.

Phase II: Stock solutions of four fungal species were inoculated into sterile saline or into pathogen-free sputum samples. AFA was applied using our optimized parameters. Our observation was that that AFA did not prove detrimental to the viability of yeast or fungal spores. No final determination could be made on the effect of AFA on hyphal elements as quantification of spike was not performed; however, plated hyphal samples did grow on SAB plates in significant numbers.

Phase III: AFA-processed sputa from CF patients grew out the same fungal organisms of interest that were noted in the clinical laboratory records.

Discussion

Phase I: A significant limitation of our study was our inability within the study timeframe to procure sputa of a sufficient volume to process a single pool under multiple conditions. Dilution provided a good compromise for our immediate goals, but ideally future studies would run diluted and non-diluted samples in parallel.

Phase II: We decided to process spores and hyphae separately to address concerns that AFA would prove too harsh a treatment for the hyphal elements that we routinely observe in fungal-positive microscopics. We processed spores or yeast cells in order to utilize their ability to be quantified by standard means (serial dilution plating). While our experiment cannot address whether there was a reduction in the hyphal elements viability due to the inability to quantify fungal balls from liquid culture, it was noted that both species in which hyphae were tested were recovered in significant number post-AFA. In Phase II-B, fungal spikes were introduced into pathogen-free sputa pools (only the growth of normal flora was recorded in the JHH Microbiology Lab record). The growth of spiked organism is consistent with the dilution factor with no adverse effect from exposure to AFA for 5 of the 6 organisms of interest; the quantification of *T. mycotoxinivorans* was not possible due to overgrowth of sample's indigenous yeast. While outside the scope of this study, it was also noted that the biofilm created by *P. aeruginosa* in one of the samples was not present on the plates inoculated with the AFA processed aliquot.

Phase III: After observing each component in relative isolation, we applied our protocol to actual clinical specimens and contrasted our results with the standard methods.

The pathogenicity of fungi within the cystic fibrosis lung is an issue of ongoing concern. The first steps in evaluating the significance of fungal species presence in CF patients, is to collect good data on what species are present. It is our hope to expand this preliminary work into a full study that would include parallel processing and plating with our current standard practices to create a more robust vision of the effect of AFA on fungal recovery from CF sputa in real time.

Conclusions

- AFA is able to quickly and reliably homogenize and liquefy mucoid sputum
- Samples in our study did not show a significant degradation of target fungal organisms
- AFA may break up biofilms caused by Pseudomonas
- Next steps would include real-time parallel testing with our clinical lab's standard practice

References

1. Kondori N, Gilljam M, Lindblad A, Jonsson B, Moore ER, Wenneras C. 2011. High rate of *Exophiala dermatitidis* recovery in the airways of patients with cystic fibrosis is associated with pancreatic insufficiency. J. Clin. Microbiol. 49:1004–1009. 10.1128/JCM.01899-10.
2. Clinical Subcommittee of the Medical/Scientific Advisory Committee of the Canadian Cystic Fibrosis Foundation. 1993. Microbiological processing of respiratory specimens from patients with cystic fibrosis. Can. J. Infect. Dis. May-June 4(3): 166-169.
3. Masoud-Landgraf L, Badura A, Eber E, Feierl G, Marth E, Buzina W. 2014. Modified culture method detects a high diversity of fungal species in cystic fibrosis patients. Med. Mycol. 52:179–186.
4. Pashley CH, Fairs A, Morley JP, Tailor S, Agbetile J, Bafadhel M, Brightling JP, Wardlaw J. 2012. Routine processing procedures for isolating filamentous fungi from respiratory sputum samples may underestimate fungal prevalence. Med. Mycol. 50:433–8.
5. Baxter CG, Jones AM, Webb K, Denning DW. 2011. Homogenisation of cystic fibrosis sputum by sonication—an essential step for Aspergillus PCR. J. Microbial Methods 85(1):75-81.

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