Mechanical Homogenization Increases Bacterial Homogeneity in Sputum

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Sputum obtained from patients with cystic fibrosis (CF) is highly viscous and often heterogeneous in bacterial distribution. Adding dithiothreitol (DTT) is the standard method for liquefaction prior to processing sputum for molecular detection assays. To determine if DTT treatment homogenizes the bacterial distribution within sputum, we measured the difference in mean total bacterial abundance and abundance of *Burkholderia multivorans* between aliquots of DTT-treated sputum samples with and without a mechanical homogenization (MH) step using a high-speed dispersing element. Additionally, we measured the effect of MH on bacterial abundance. We found a significant difference between the mean bacterial abundances in aliquots that were subjected to only DTT treatment and those of the aliquots which included an MH step (all bacteria, $P = 0.04$; *B. multivorans*, $P = 0.05$). There was no significant effect of MH on bacterial abundance in sputum. Although our results are from a single CF patient, they indicate that mechanical homogenization increases the homogeneity of bacteria in sputum.

Sputum samples are regularly used in DNA-based studies to determine the microbial environment found in the lungs of patients with cystic fibrosis (CF). Expectorated sputum is provided by the patient, a portion is processed, and the remainder is stored in a freezer. Since sputum can be thick and difficult to process, the viscosity is reduced through chemical processing (CP) prior to aliquot removal for use in an assay. The most common CP method for molecular detection assays (1) includes mixing a ratio of sputum to dithiothreitol (DTT), commonly known as Sputasol, Sputolysin, or Cleland's reagent (2). While adding DTT prior to aliquoting is the preferred procedure, sputum is often processed after an aliquot has been removed (3, 4). Although CP has been shown to be useful for reducing sputum viscosity (5), it is not clear how effective this method is for homogenizing and evenly distributing bacterial cells, which may be clumped in sputum (6).

As part of another study, we measured the total bacterial abundance in >100 sputum samples collected over 3 years from a single CF patient, and we observed >1,000-fold variations among the samples. Such a wide range in bacterial abundance can arise from variations in the location in the lungs from which the sputum was expectorated (7) and from assaying aliquots not representative of the sputum sample. We expect that if aliquots are not representative, the viscous nature of sputum may be causing bacterial heterogeneity, and we consider here whether mechanical homogenization (MH), as described below, should be added to sampling protocols. The efficacy of this additional processing step has not been evaluated.

Here, we measured bacterial abundance in DTT-treated sputum to determine if MH decreases the variability in abundance of all bacteria and in the abundance of *Burkholderia multivorans* in multiple aliquots of sputum. We also used a descriptive statistic to determine if mechanical disruption of sputum using a high-performance disperser affects bacterial recovery.

MATERIALS AND METHODS

Samples. Nine expectorated sputum samples (designated 1 to 9) were obtained from a CF patient (institutional review board protocol approval 11-12-36) on separate days over an 18-week period. The patient was clin-

ically stable, as judged by a treating physician, and was administered only prophylactic antibiotic treatment during the time of collection. To improve the temporal linkage, the samples were collected each morning. The patient expectorated sputum into a 15-ml Falcon tube that was then placed on ice during transport to the lab and processed immediately. The sputum color was noted, but no relationship was identified between the color and heterogeneity of the sample. Other physical properties of our sputum samples, such as viscosity, were not reported due to the lack of a consistent measure for those properties; however, we did note that we were unable to pipette any of the samples until after the addition of DTT.

Aliquot size and storage temperature. The results obtained in the study were based on our use of 400- μ l aliquots according to the manufacturer's recommendation for the DNA extraction kit; the volume of sputum for the majority of samples collected in this study was approximately 1 ml. We immediately processed the sputum samples collected from the CF patient to ensure that no other factors, such as storage time or temperature, would affect their characteristics or composition.

Chemical processing. Each sputum sample was mixed at a 1:3 ratio of sputum to 0.1% dithiothreitol solution, vortexed vigorously, and then incubated at 37°C for 1 h.

Mechanical homogenization. Sputum was subjected to MH for 2 min using a high-performance dispersing instrument (IKA Ultra-Turrax-25 digital homogenizer; Staufen, Germany) set to 12,000 rpm. The metal shaft of the disperser was disinfected between each sample fraction using a combination of steps, which included placing the shaft in a 5% bleach solution and then 70% ethanol for at least 30 s each. The shaft was then rinsed thoroughly with deionized distilled water. All aliquots were weighed and then stored on ice until the DNA extraction.

DNA extraction. DNA was extracted from each sputum aliquot using the IT 1-2-3 VIBE sample purification kit (Biofire Diagnostics, Inc., Salt Lake City, UT), and its concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). All ex-

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FIG 1 Flow chart of the experimental design. Tubes with large dots represent sputum without mechanical homogenization. Filled tubes represent sputum with mechanical homogenization.

tracted DNA was immediately stored at -20°C until its use for quantitative PCR (qPCR).

Quantitative PCR. qPCR was used to enumerate the total amount of bacteria and that of a species of a low-abundance taxon, *Burkholderia multivorans*. Targeting a low-abundance taxon, whose distribution may be less uniform than that of all bacteria, may better reveal the ability of MH to increase homogeneity. The qPCR mixture contained 10 μl of Perfecta SYBR green FastMix reagent, low ROX (Quanta Biosciences, Gaithersburg, MD), 0.5 μl of 100 pmol/ μl of each primer, 5 μl of DNA, and 4 μl of nuclease-free water to a final volume of 20 μl . Universal primers (8) were used to target the 16S rRNA gene sequence with an expected fragment size of 466 bp and to measure the abundance of all bacteria in the sample. *Burkholderia cepacia* complex (Bcc)-specific primers (9) were used to target *B. multivorans*, the only species of *Burkholderia* colonizing our patient, and to generate a fragment of 333 bp. qPCR was performed using the ABI 7500 fast real-time PCR system (Applied Biosystems, Carlsbad, CA) with an initial step of 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C . Melting curves were determined following the qPCR by 1 cycle of 15 s at 95°C , 1 min at 60°C , 30 s at 95°C , and 15 s at 60°C . Standard curves were created for each primer pair using 10-fold dilutions of amplicons generated using an *Escherichia coli* strain as the DNA template for the 16S rRNA gene sequence primers and *B. multivorans* for the Bcc-specific primers. The DNA copy number per gram of sputum was calculated for each sample based on a standard curve with a 1×10^5 -fold linear range in threshold cycle (C_T) values. To ensure quality and consistency, three technical replicates were performed, and Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were followed for all qPCRs (10).

Statistical analysis of bacterial abundance between groups of homogenized and nonhomogenized aliquots. Each of nine sputum samples were divided into two equal fractions based on their weight and labeled A and B (Fig. 1). Each fraction was then divided into two groups of six aliquots. Fraction A was divided to determine if MH has an effect on the difference in means of abundance between nonhomogenized (group A1, 2 aliquots) and homogenized (group A2, 4 aliquots) sputum. Fraction B was also divided into two groups of 6 aliquots, and all of the aliquots were subjected to MH; group B1 (2 aliquots) and group B2 (4 aliquots) were designed as homogenization control variables for comparison to groups A1 and A2, respectively. We selected only two 400- μl aliquots for groups A1 and B1 because the total volume of sputum was different for each sample, and at the beginning of the experiment, we did not know whether

there would be sufficient volume to obtain six 400- μl aliquots from each fraction. By choosing 2 aliquots for groups A1 and B1, we were sure to collect the minimum number needed for our statistical tests when comparing the difference in means between groups. To determine if the aliquots from each fraction were representative of the whole sample, a two-way between-subjects analysis of variance (ANOVA) was performed for each fraction.

The two independent variables were designated according to each fraction; the variables in fraction A represent the differences in means with no homogenization, and the variables in fraction B represent the differences in means with homogenization, with means expressed as the $\log_{10}$ 16S copy number (total bacterial abundance) or $\log_{10}$ *B. multivorans* copy number (*B. multivorans* abundance). We compared groups A1 with A2 to determine if there was a difference in means resulting from homogenization. We compared groups B1 with B2 to determine how much of an observed difference between groups A1 and A2 was independent of homogenization. The R programming language was used with the following model to perform the two-way between-subjects ANOVA and compare the difference in mean bacterial abundance using each fraction as a function of the nine samples, with each group of aliquots (A1, A2, B1, and B2) as the interaction terms:

$$Y_i = H_0 + H_1X_i + e_i \quad (1)$$

If we hypothesize that H_0 indicates no difference in the means, the full equation becomes

$$Y_i = H_1(\text{sample}) + H_2(\text{fraction}) + H_3(\text{sample} \times \text{fraction}) + e_i \quad (2)$$

where e_i represents the residuals.

We used this model and a null hypothesis to compare aliquot groups from each fraction. We interpreted a P value of <0.05 as a significant difference in the means and a P value of >0.05 as no significant difference in the means. Using this model, we sought to determine if mechanically homogenizing sputum prior to removing aliquots has an effect on the difference in bacterial abundance between the aliquot groups.

Effect of MH on bacterial abundance. A meta-analysis was used to summarize the effect of MH on the abundance of all bacteria and *B. multivorans* in each sample. The Cohen d effect size (8) measure was used by treating each sputum sample as independent of one another (4). The aliquots were separated and treated as two separate groups (with and without MH) within each sputum sample, and the number of aliquots

TABLE 1 Effect of mechanical homogenization on the abundance of all bacteria and that of *B. multivorans* in nine sputum samples

Sample no.	Total bacterial abundance			<i>B. multivorans</i> abundance		
	δ^a	V^b	$SEM\delta^c$	δ	V	$SEM\delta$
1	-0.24	0.61	0.32	-0.22	0.61	0.32
2	0.25	0.61	0.32	-0.16	0.61	0.32
3	-1.32	0.60	0.32	-0.92	0.60	0.32
4	0.52	0.61	0.32	0.80	0.62	0.32
5	-2.04	0.60	0.32	-1.27	0.60	0.32
6	-1.00	0.62	0.32	-0.83	0.62	0.32
7	0.33	0.62	0.34	-1.50	0.63	0.34
8	1.44	0.60	0.32	1.80	0.61	0.32
9	-0.20	0.61	0.32	-1.03	0.61	0.32

^a Effect size.^b Variance of each sample.^c Standard error of the mean of the effect size.

within each group was the sample size. Cohen (11) provides a guideline, if necessary, for interpreting the effect size by stating that a δ value of 0.20 is a small effect, a δ value of 0.50 is a medium effect, and a δ value of ≥ 0.80 is a large effect. Table 1 shows the effect size on total bacterial abundance and *B. multivorans* for each of the nine sputum samples. Since the effect size was quite variable from one sample to the next, we calculated the overall effect size for total bacterial abundance and *B. multivorans* abundance using a method similar to that of Rogers et al. (4), in which the overall effect size (E) is weighted by the variance (V) of each sample. We then calculated the standard error of the mean for the combined samples (SEM_E):

$$E = \frac{\sum_{i=1}^9 \frac{1}{V_i} \times \delta_i}{\sum_{i=1}^9 \frac{1}{V_i}} \quad (3)$$

$$SEM_M = \frac{\sqrt{\frac{1}{\sum_{i=1}^9 \frac{1}{V_i}}}}{\sqrt{9}} \quad (4)$$

RESULTS

Determining if DTT treatment is sufficient to homogenize a sputum sample may be accomplished by comparing the bacterial abundances in sputum aliquots taken without and then with DTT treatment. Because of the high viscosity of untreated sputum, however, this strategy was not possible in this study. Our alternative approach was to examine whether adding a mechanical homogenization step to DTT-treated sputum would change the abundance distribution of total bacteria, as well as of one species of a low-abundance taxon, *B. multivorans*. If MH decreases the difference in the means of abundance between aliquots, then DTT treatment alone does not homogenize sputum.

The abundances of total bacteria and of *B. multivorans* are presented in a box plot for each of the nine sputum samples (Fig. 2). Our two-way ANOVA model showed a significant difference in the means of total bacterial abundance ($F = 2.32$, $P = 0.04$) and of the abundance of *B. multivorans* ($F = 2.20$, $P = 0.05$) between the A1 (no MH) and A2 (with MH) aliquot groups in fraction A (Fig. 2A and C). For the parallel control aliquots in fraction B (all with MH), we found no significant differences in the means of total bacterial abundance ($F = 0.612$, $P = 0.76$) or of the abun-

dance of *B. multivorans* ($F = 0.20$, $P = 0.99$) between the B1 and B2 aliquot groups (Fig. 2B and D).

The absolute mean values for total bacterial abundance, expressed as the 16S rRNA gene copy number per gram sputum, were 9.74e9, 9.82e9, 9.78e9, and 9.71e9 for fractions A1, A2, B1, and B2, respectively. The absolute mean values for *B. multivorans* abundance were 6.88e6, 6.95e6, 6.91e6, and 7.00e6 for fractions A1, A2, B1, and B2, respectively. These results indicate that MH increases the homogeneity of sputum.

Adding the MH step, in which we used a high-speed dispersal element to mechanically homogenize the sample, may potentiate a confounding variable by changing the efficiency of the DNA extraction process. If MH were to affect extraction efficiency, we would expect to see a larger difference between our mechanically and nonmechanically homogenized aliquots, not because of the homogenization of the starting material but because of improved DNA extraction, reflected by an increase in bacterial abundance. As a result, we examined the effect of MH on bacterial abundance by using the Cohen d effect size analysis of total bacterial and *B. multivorans* abundance, as shown by Rogers et al. (4). By applying the Cohen d effect size analysis, we can compare the bacterial abundances between all aliquots and determine whether MH affects DNA-based analyses such as qPCR. As mentioned above, Cohen provides a guideline for interpreting effect size with a scale ranging from a small effect (≤ 0.20) to a large effect (≥ 0.80), with each value representing the number of standard deviations from the mean. The effect of MH on bacterial abundance varied among our sputum samples (Table 1), from -0.20 to -2.04 for total bacterial abundance and from -0.16 to 1.80 for *B. multivorans* abundance (Fig. 3). Because of the range of individual values from our samples, including those that indicated a large effect, we calculated overall effect sizes of -0.25 and -0.37 for total bacterial abundance and *B. multivorans* abundance, respectively (Fig. 4). While Cohen cautioned against overinterpretation of the effect size, the individual effect size values suggest that MH can affect bacterial abundance. However, as Rogers et al. noted, given that the standard error of the overall effect size values cross over zero, we suggest that MH does not have a significant effect on bacterial abundance.

DISCUSSION

The method of processing sputum from CF patients varies from one study to the next (12–14). Dithiothreitol has been recommended for use as an agent to liquefy sputum since 1955 (15). Additional liquefaction steps, such as mechanical homogenization, have been used in studies examining bacteria in sputum, but these are not routinely performed, and their efficacies have not been examined (13). Because most standard protocols for DNA extraction, such as using the VIBE 1-2-3 sample purification kit (as in this study), require a volume of sputum smaller than that which may be expectorated from an adult with CF, most labs will use only an aliquot of a sputum sample for culture-independent assays and store the remainder. Since the bacteria in individual sputum samples are heterogeneously distributed (16), including cells clustered in biofilm, incomplete homogenization may result in the collection of an aliquot that does not represent the whole sputum sample. Here, we examined the aliquot representativeness of sputum samples.

Intrasample bacterial heterogeneity is reduced with mechanical homogenization. The high viscosity of sputum pre-

FIG 2 Effect of MH on heterogeneity of total bacterial abundance and *B. multivorans* abundance among aliquots of nine sputum samples. Comparison of A1 (without MH) aliquots to A2 (with MH) aliquots from fraction A shows a significant difference in the means of abundance in all bacteria ($P = 0.04$) (A) and in *B. multivorans* ($P = 0.05$) (C). Comparison of B1 aliquots to B2 aliquots (both with MH) from fraction B shows no significant difference in the means of abundance in all bacteria ($P = 0.76$) (B) and in *B. multivorans* ($P = 0.99$) (D).

vented us from directly comparing aliquots of sputum with DTT treatment and those without it. DTT is effective for liquefying sputum, but samples with high viscosity remain difficult to pipette even after this chemical treatment. We therefore removed DTT as a variable in our analysis by adding it to all samples, and we asked whether adding a mechanical homogenization step would change the distributions of total bacterial abundance and *B. multivorans* abundance.

Sputum expectorated by cystic fibrosis patients can vary in viscosity and bacterial concentration for a number of reasons, including patient hydration at the time of collection, the location in the lungs from which it originates, and simple contamination from passing through the oropharyngeal cavity (3, 7). The bacterial distribution we examined was between groups of aliquots (with and without MH) taken from each of nine sputum samples. This experimental design eliminated intersample variability.

A two-way ANOVA revealed a significant decrease in the difference in mean bacterial abundance between groups of aliquots of sputum for both total bacteria and *B. multivorans* (Fig. 2). These results indicate that using the high-performance disperser increases the distribution of bacteria in the sputum.

Effect of MH on bacterial abundance. Mechanical homogenization may simply disperse bacteria or may also improve extraction efficiency by mechanically breaking up the bacterial clumps or freeing cells from the exopolysaccharide alginate (6). These two models can be differentiated by determining if MH leads to an increase in the total amount of bacteria recovered. We observed differences in effect sizes but did not identify any discernible patterns among the sputum samples (Fig. 2). Based on Cohen's guidelines (see Results), we may conclude that MH had a large effect on the total bacterial abundance of some sputum samples and a small effect on that of others. However, as mentioned above,

FIG 3 The effect of mechanical homogenization on the abundance of total bacteria (A) and of *B. multivorans* (B). The abundances were measured by qPCR for paired groups of aliquots with and without MH, as described in Materials and Methods. The columns represent the Hedges *d* effect sizes of mechanical homogenization on bacterial recovery. The error bars were determined by the SEM of the effect size (δ). Error bars that cross zero indicate no effect.

Cohen cautioned against the use of these guidelines as a universal tool, since the context of effect size can vary based on the experiment. Given the overall effect size of MH on total bacterial abundance and on *B. multivorans* abundance and the standard error of the mean for each, MH likely has little effect on abundance (Fig. 3) and therefore would not result in a significant decrease or increase in the recovery of bacteria in sputum.

Conclusion. Mechanical disruption is not a new method for processing sputum (17); however, to the best of our knowledge, its impact on decreasing the innate heterogeneity of highly viscous sputum has not been reported. We found that mechanical disruption of the sputum dramatically increased sputum bacterial homogeneity without affecting the bacterial abundance. We recommend adding mechanical disruption as a step in the sputum-processing protocol to increase the representativeness of sample aliquots and ensure consistency in downstream analysis of any further aliquots obtained from the same sputum sample.

While we recognize the statistical limitations of analyzing samples collected from a single patient, we do not expect the bacterial heterogeneity we observed in CF sputum to be patient specific. Bacterial heterogeneity is likely due to the composition and viscosity of sputum. In addition, samples collected from this single

patient were sufficient to demonstrate our protocol for mechanically homogenizing sputum.

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FIG 4 The overall effect of mechanical homogenization on the abundance of total bacteria and *B. multivorans* in sputum. The abundances were measured by qPCR for paired groups of aliquots with and without MH. The columns represent the effect sizes of mechanical homogenization on bacterial recovery. The error bars were determined by the SEM of the overall effect size (δ). Error bars that cross zero indicate no effect.

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