Peritoneal Effluent Cell-Free DNA Sequencing in Peritoneal Dialysis Patients With and Without Peritonitis

Philip Burnham, Fanny Chen, Alexandre P. Cheng, Vesh Srivatana, Lisa T. Zhang, Emmanuel Edusei, Shady Albakry, Brittany Botticelli, Xunxi Guo, Amanda Renaghan, Jeffrey Silberzweig, Darshana M. Dadhania, Joan S. Lenz, Michael Heyang, Iliyan D. Iliev, Joshua A. Hayden, Lars F. Westblade, Iwijn De Vlaminck, and John R. Lee**

Rationale & Objective: Conventional culture can be insensitive for the detection of rare infections and for the detection of common infections in the setting of recent antibiotic usage. Patients receiving peritoneal dialysis (PD) with suspected peritonitis have a significant proportion of negative conventional cultures. This study examines the utility of metagenomic sequencing of peritoneal effluent cell-free DNA (cfDNA) for evaluating the peritoneal effluent in PD patients with and without peritonitis.

Study Design: Prospective cohort study.

Setting & Participants: We prospectively characterized cfDNA in 68 peritoneal effluent samples obtained from 33 patients receiving PD at a single center from September 2016 to July 2018.

Outcomes: Peritoneal effluent, microbial, and human cfDNA characteristics were evaluated in culture-confirmed peritonitis and culture-negative peritonitis.

Analytical Approach: Descriptive statistics were analyzed and microbial cfDNA was detected in culture-confirmed peritonitis and culture-negative peritonitis.

Results: Metagenomic sequencing of cfDNA was able to detect and identify bacterial, viral, and eukaryotic pathogens in the peritoneal effluent from PD patients with culture-confirmed peritonitis, as well as patients with recent antibiotic usage and in cases of culture-negative peritonitis.

Limitations: Parallel cultures were not obtained in all the peritoneal effluent specimens.

Conclusions: Metagenomic cfDNA sequencing of the peritoneal effluent can identify pathogens in PD patients with peritonitis, including culture-negative peritonitis.

Complete author and article information provided before references.

Correspondence to I. De Vlaminck (vlaminck@cornell.edu) or J.R. Lee (jrl2002@med.cornell.edu)

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Conventional culture can be analytically insensitive for the detection of rare infections and for the detection of common infections in the setting of recent antibiotic usage. Indeed, patients with end-stage kidney disease on peritoneal dialysis (PD) have negative conventional cultures in 20% of suspected peritonitis cases.[1] It is unclear whether the large proportion of unresolved cases is due to recent antibiotic use interfering with culture, infectious processes beyond peritonitis, infections due to fastidious or nonculturable organisms, or noninfectious processes.

We have recently demonstrated a "digital culture" test, based on shotgun metagenomic DNA sequencing of cell-free DNA (cfDNA), that can detect a range of bacterial, viral, and fungal infections.[2,3] Importantly, we have applied this technique to body fluids that have been classically considered sterile, such as urine and amniotic fluid.[4–6] Here, we investigate the utility of metagenomic sequencing of cfDNA in peritoneal effluent to monitor infections in PD patients.

METHODS

Description of the Study Cohort

From September 2016 to July 2018, we recruited PD patients with and without peritonitis on a continual basis for the collection of serial peritoneal effluent specimens at New York-Presbyterian Hospital/Weill Cornell Medical Center and The Rogosin Institute. The Weill Cornell Institutional Review Board approved the study (no. 1604017181), and each patient provided written informed consent. The research study was conducted according to the Declaration of Istanbul. We collected serial peritoneal effluent specimens from PD patients with clinical peritonitis (peritonitis group) and also serial peritoneal effluent specimens from PD patients who did not have clinical evidence of peritonitis in the inpatient and outpatient setting (no-peritonitis group). Peritonitis was defined using the International Society of Peritoneal Dialysis (ISPD) 2016 guidelines and was diagnosed using at least 2 of the following criteria: (1) abdominal pain and/or cloudy dialysis effluent; (2) dialysis effluent white cell count >100 per microliter with >50% polymorphonuclear white blood cells; and (3) positive dialysis effluent culture.[7]

Peritoneal Effluent Collection

Peritoneal effluent specimens were collected by drainage of fluid from the peritoneal catheter in PD patients, and at least 30 mL was collected. The peritoneal effluent specimen was subsequently centrifuged at 2,000 × g for 30 minutes and 1-mL aliquots of peritoneal effluent supernatants were collected and stored at -80 °C.

Cell-Free DNA Extraction

The 1-mL aliquots of peritoneal effluent were thawed from -80 °C storage and were centrifuged at 1,500 × g for 5 minutes. Then, 900 μL of the supernatant was transferred

PLAIN LANGUAGE SUMMARY

Patients on peritoneal dialysis (PD) unfortunately experience peritonitis, which is commonly caused by an infection in their abdomen. Culturing the peritoneal effluent from the abdomen can usually identify the microbe suspected of causing the infection, but in some cases is unable to do so because of the limitation of the culturing assay. In this study, we examined the utility of metagenomic sequencing of peritoneal effluent cell-free DNA in PD patients with and without peritonitis. We found that the cell-free DNA sequencing assay is able to detect and identify bacterial, viral, and eukaryotic pathogens in the peritoneal effluent from PD patients with culture-confirmed peritonitis, as well as those with culture-negative peritonitis.

to a new tube and 100 μL of sterile 1x phosphate-buffered saline was added. The supernatant was processed using the "Plasma Supernatant 1 mL" protocol of the QiaAmp circulating nucleic acid extraction kit. The sample was eluted into 30 μL of nuclease-free water and the DNA concentration was measured using the Qubit 3.0 fluorometer (HS DNA kit). DNA samples were stored at -20 °C until library preparation. A negative control, previously described,[5,8] was processed in the extraction, library preparation, and sequencing steps.

Library Preparation and Next-Generation Sequencing

We processed 12 μL to 26 μL of a sample using a single-stranded DNA library preparation protocol without alteration.[8] Samples were indexed with 5 to 15 cycles of polymerase chain reaction amplification. DNA libraries were characterized using the Advanced Analytical Technologies Inc (AATI) fragment analyzer to confirm the absence of polymerase chain reaction primer dimers. DNA libraries were then sequenced on a NextSeq 500 using a paired-end, 2 × 75 bp kit with the substitution of Read 1 sequencing primer with the sequence (5'-ACACTCTTTCCCTACACG ACGCTCTTCC-3'). For 18 samples, a technical replicate was processed. An average of 50.7 million reads were sequenced for each sample or replicate. For 36 of the 68 samples, we detected a primer sequence used for experiments in the laboratory (Item S1); these adaptors were not overly abundant (of samples with primer, the median primer abundance was 0.004% of total reads), and were automatically removed in the low biomass background correction (LBBC) pipeline.[4]

Processing Next-Generation Sequencing Data

Raw sequencing reads were analyzed for low-quality reads and Illumina-specific sequences; poor-quality sequences were removed. Low-read-quality sequence ends were trimmed. Filtered reads were aligned against the human reference genome (UCSC hg19; www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/) using BWA-MEM.[9] Human-aligned reads were analyzed for fragment length and sequencing coverage. Nonhuman reads were converted back into paired FASTQ format.

Peritoneal Effluent Microbiome Analysis

Nonhuman reads were aligned against a curated reference database as previously described[3] using National Center for Biotechnology Information (NCBI) BLAST[10] in a single read fashion. The aligned microbial taxa for each read in pairs were compared, and reads were removed if pairs did not align to the same microbe or if read pairs were too far apart (>5 kbp). Read lengths were determined for each microbe in a sample to generate plots in Fig 1. Filtered BLAST-aligned reads were then merged (so read pairs were transformed to single reads) and a maximum likelihood estimator was used to estimate microbial abundance by taxa.[11] A LBBC algorithm[4] was used to remove physical and digital contaminants using the following constraints: bacteria and eukaryotes (filter at tax_ID level; coefficient of variation filter = 2.5; batch variation filter = -7; negative control multiplier filter = 50) and viruses (filter at tax_ID level; no coefficient of variation filter; batch variation filter = -8; negative control multiplier filter = 50). All samples and replicates were included in the background correction pipeline.

Antibiotic-Resistant Gene Analysis

Paired nonhuman FASTQ files were analyzed using the Resistance Gene Identifier Program using the Comprehensive Antibiotic Resistance Database v3.1.3 and CARD Resistomes & Variants v 3.0.9.[12] The FASTQ files were run using the rgi bwt command using the bowtie2 aligner and including wildcard.

Peritoneal Effluent Cell Culture

Peritoneal effluent specimens that were collected for clinical indications were cultured at Spectra Laboratories (Rockleigh, NJ) per their standard operating procedures or at New York-Presbyterian Hospital/Weill Cornell Medical Center per their standard operating procedures. Peritoneal effluent specimens which were not collected for clinical indications were cultured at New York-Presbyterian Hospital/Weill Cornell Medical Center using a combination of broth and liquid media. Peritoneal effluent was inoculated into aerobic and anaerobic blood culture vials (Becton, Dickinson, and Company [BD]) and incubated in a continuous automated blood culture instrument (BACTEC FX; BD). Specimens were also plated on tryptic soy agar with 5% sheep blood, MacConkey agar, and chocolate agar, and inoculated into thioglycolate broth (BD and Hardy Diagnostics). These media were incubated at 35 °C in 5% carbon dioxide. Finally, a portion of the fluid was inoculated onto Brucella Blood agar (Anaerobe Systems) and incubated under anaerobic conditions. Identification of microorganisms isolated in culture was performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker Daltonics, Inc) and antibiotic

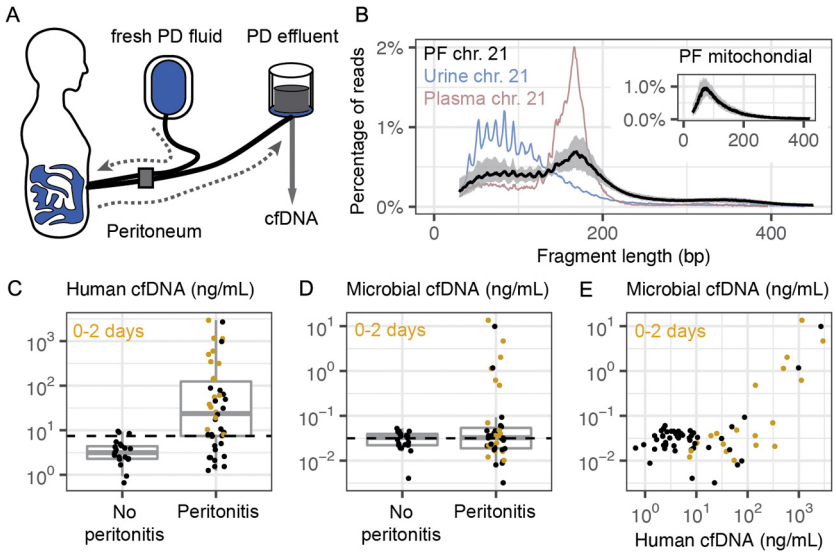

Figure 1. (A) Comparison of unfiltered cell-free DNA (cfDNA) biomass (ng/mL peritoneal effluent) for various culture-confirmed organisms present in peritoneal effluent. Biomass of culture-confirmed organisms in patients 0 to 2 days after diagnosis (and the first sample over that time period) compared to samples with negative culture. Dotted lines show median values of organism biomass in 10 samples with negative cultures. (B) Heatmap of cfDNA of common bacterial species by peritonitis group or no-peritonitis group specimens. On the y axis are peritonitis group cases by diagnosis (top 11) and no-peritonitis cases confirmed by culture (bottom 10), and on the x-axis are bacterial species in the peritonitis group. The color represents the species biomass by intensity.

susceptibility testing was performed using broth microdilution (Beckman Coulter, Inc).

Statistical Analyses

All statistical analyses were performed using the R Program (R 3.3.3). Correlation measurements were performed using Pearson or Spearman correlations (function *cor.test*). The distribution of continuous variables was analyzed using the 2-tailed Wilcoxon rank sum test and the distribution of categorical variables was analyzed using the Fisher exact test.

Data Availability

Sequencing data that support the findings of this study will be made available in the database of Genotypes and Phenotypes (dbGaP) phs002251.v1.p1. Urinary cfDNA used in data presented in Fig. 1 are available at dbGaP phs001564.v3.p1. Local institutional review board approval will be needed to access the data.

RESULTS

Characteristics of Peritoneal Effluent cfDNA in PD Patients

We isolated cfDNA from 1 mL of peritoneal effluent obtained from 68 specimens collected from 33 PD patients (Fig. 1A). Among the 33 PD patients, 17 were suspected of having clinical peritonitis and provided 46 specimens before, during, and after the suspected clinical peritonitis episode (peritonitis group), while 16 patients did not have evidence of clinical peritonitis and provided 22 specimens (no-peritonitis group). Clinical characteristics of the peritonitis group and the no-peritonitis group are shown in Table 1. Within the peritonitis group, 13 patients had culture-confirmed peritonitis and 4 patients had culture-negative peritonitis.

Libraries were sequenced on the Illumina NextSeq platform and we obtained 57.2 ± 18.7 million sequences per sample; on average, 72.1% of paired-end reads aligned to the human genome. We compared the fragmentation profile of cfDNA in peritoneal effluent to the fragmentation profiles of cfDNA in urine and plasma, measured using the same DNA sequencing and analysis protocol (Fig. 1B). As previously reported,[8,13] plasma cfDNA is protected by nucleosome binding and most cfDNA fragments are longer than 100 bp (median, 82.7%; n = 8 samples; Fig. 1B), while urinary cfDNA is more degraded, with fewer fragments longer than 100 bp (median, 44.8%; n = 18).[6,14] We found that cfDNA extracted from peritoneal effluent, in contrast, shared properties associated with both plasma and urinary cfDNA and was a mixture of both long and short fragments (73.8% with length >100 bp; n = 68; Fig. 1B).

Role of Peritoneal Effluent cfDNA in Distinguishing Culture-Confirmed Peritonitis From No Peritonitis

We compared the concentrations of host-derived peritoneal effluent cfDNA and microbial-derived cfDNA in the peritonitis group and the no-peritonitis group (Fig. 1C-D). The concentration of host cfDNA was significantly higher in specimens within 2 days of presentation in the peritonitis group than specimens from the no-peritonitis group ($P = 2.5 \times 10^{-10}$; Wilcoxon rank sum test). The concentration of microbial-derived cfDNA was not significantly different between specimens within 2 days of presentation in the peritonitis group and specimens from the no-peritonitis group ($P = 0.34$; Wilcoxon rank sum test). We also found that the concentrations of microbial-derived cfDNA and host-specific cfDNA were highly correlated for samples with more than 20 ng/mL of host-specific cfDNA (Spearman $\rho = 0.75$; $P = 4.5 \times 10^{-5}$; n = 23; Fig. 1E). To test the ability of cfDNA to monitor

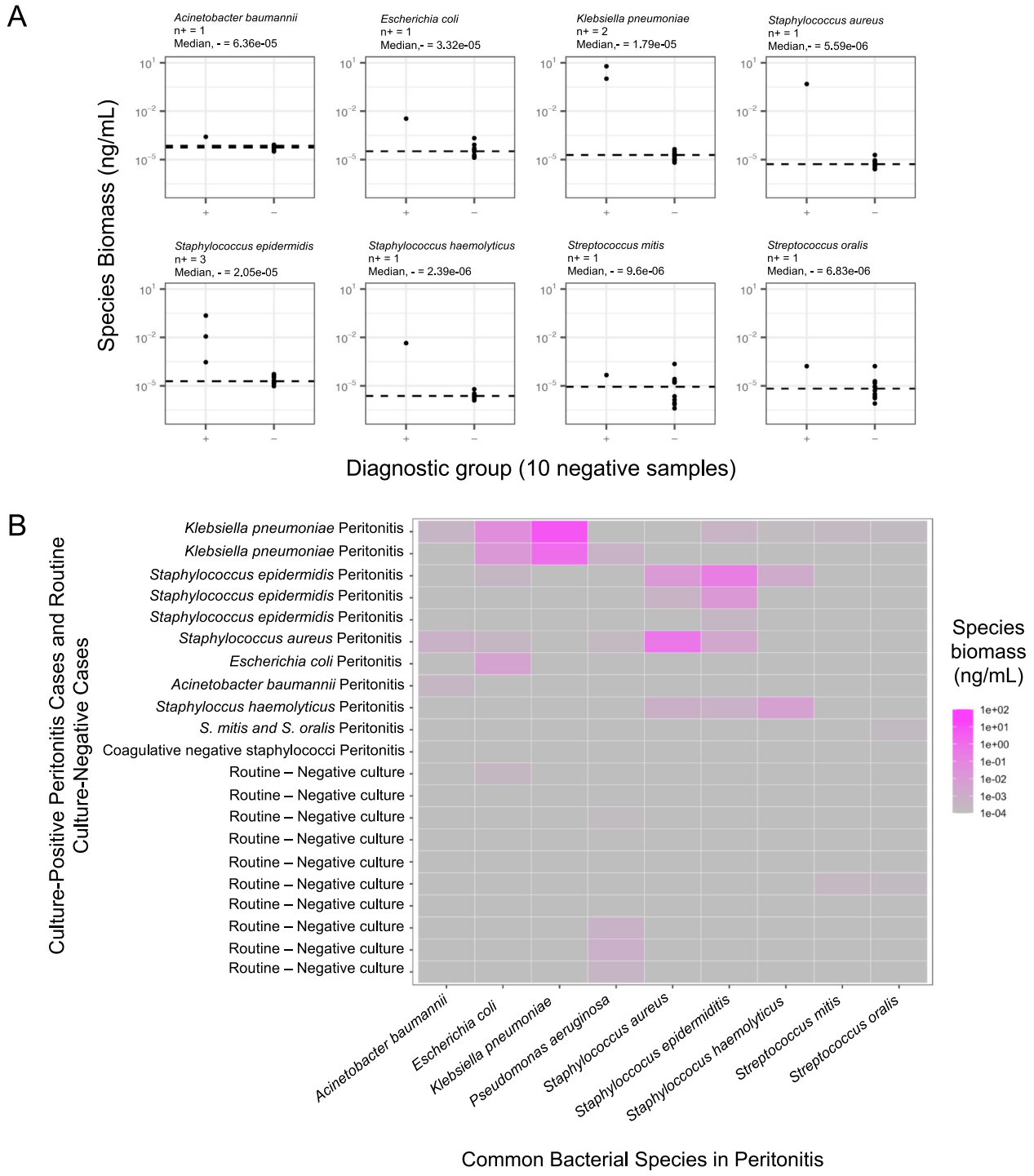

Figure 2. Peritoneal effluent from peritoneal dialysis (PD) patients contains cell-free DNA (cfDNA) from both human and microbial sources in cases of peritonitis. (A) Schematic illustration showing the exchange of peritoneal effluent and the collection of peritoneal effluent to recover cfDNA. (B) Fragment length distributions of cfDNA from peritoneal effluent (black) compared to cfDNA fragmentation patterns from urine (blue) and plasma (lavender). Inset: fragment length distribution of PF cfDNA aligned to the mitochondrial genome. (C) Concentration of human-derived cfDNA is shown for peritoneal effluent specimens obtained from patients in the peritonitis group and patients in the no-peritonitis group. (D) Concentration of microbial-derived cfDNA is shown for peritoneal effluent specimens from patients in the peritonitis group and patients in the no-peritonitis group. (E) Concentration of microbial and human cfDNA is compared for all samples (n = 68). (C-E) Samples collected within 0 to 2 days of peritonitis diagnosis are indicated. Abbreviations: chr., chromosome; PF, peritoneal effluent.

Table 1. Clinical Characteristics of the Peritonitis Group and the No-Peritonitis Group

Characteristic	Peritonitis Group (n = 17)	No-Peritonitis Group (n = 16)	*P* Value
Age in years, median (IQR)	54 (42-63)	58 (52-69)	0.35
Female gender	11 (65%)	9 (56%)	0.73
Race			
White	6 (35%)	6 (38%)	0.99
Black	5 (29%)	5 (31%)	0.99
Asian	1 (6%)	2 (13%)	0.60
American Indian	0 (0%)	1 (6%)	0.48
Other	5 (29%)	2 (13%)	0.40
Hypertension	17 (100%)	14 (88%)	0.23
Diabetes mellitus	9 (53%)	6 (38%)	0.49
Hyperlipidemia	11 (65%)	7 (44%)	0.30
Type of dialysis			
CAPD	5 (29%)	5 (31%)	0.99
CCPD	12 (71%)	11 (69%)	0.99
Years receiving PD, median (IQR)	1.4 (0.7-2.4)	2.1 (1.3-3.5)	0.21
Prior peritonitis episodes, median (IQR)	0 (0-2)	0 (0-0.3)	0.19
White blood cell count, 10^3/uL, median (IQR)	8.2 (6.7-9.0)	7.9 (6.3-8.9)	0.73
Hemoglobin, g/dL, median (IQR)	9.6 (9.1-10.8)	11.6 (10.4-12.3)	0.03
Platelets, 10^3/uL, median (IQR)	275 (198-335)	230 (180-273)	0.39
Sodium, mmol/L, median (IQR)	132 (130-136)	134 (132-136)	0.70
Potassium, mmol/L, median (IQR)	3.9 (3.5-4.3)	4.0 (3.8-5.0)	0.42
Bicarbonate, mmmol/L, median (IQR)	26 (23-27)	27 (26-28)	0.12
Blood urea nitrogen, m/dL, median (IQR)	51 (45-67)	56 (37-58)	0.83
Creatinine, mg/dL, median (IQR)	10.0 (7.8-12.3)	9.1 (7.6-12.5)	0.97
Calcium, mg/dL, median (IQR)	8.6 (8.4-9.0)	9.1 (8.8-9.9)	0.02

Note: P values were calculated using a Wilcoxon signed rank test or Fisher exact test. All laboratory values were obtained at the time of the first peritoneal effluent specimen collection.
Abbreviations: CAPD, continuous ambulatory peritoneal dialysis; CCPD, continuous cycling peritoneal dialysis; IQR, interquartile range; PD, peritoneal dialysis.

peritonitis, we examined the temporal dynamics of the host and microbial cfDNA concentrations relative to the day of peritonitis onset. We found that, in general, host and microbial cfDNA concentrations were elevated at the day of diagnosis and up to 2 days following antibiotic treatment. However, both returned to baseline amounts after several days (Fig S1A and B).

We also found a strong correlation between library sequencing quality (as determined by unique read fraction) and the measured concentration of peritoneal effluent cfDNA (Spearman $\rho = 0.888$; $P < 10^{-15}$). Given the possibility of environmental microbial DNA contamination,[4,15] we evaluated cfDNA in the most common peritoneal dialysate solutions as negative controls (Dianeal 1.5%, Dianeal 2.5%, and Dianeal 4.25%; Baxter). These samples had similar characteristics to peritoneal effluent specimens with concentrations below 20 ng/mL and had a median cfDNA concentration of 8 ng/mL and a low number of unique reads (median, 11.1%). The scarcity of cfDNA in these 3 controls suggests that environmental contamination sets the limit of detection in samples with a low total DNA biomass.[15]

Identification of Pathogens by Peritoneal Effluent cfDNA in Both Culture-Positive Peritonitis and Culture-Negative Peritonitis

Among the peritonitis group, there were 11 culture-confirmed peritonitis events in which PD patients provided peritoneal effluent specimens within 2 days of diagnosis. These cases included: 3 *Staphylococcus epidermidis*, 2 *Klebsiella pneumoniae*, 1 *Escherichia coli*, 1 *Staphylococcus haemolyticus*, 1 *Staphylococcus aureus*, 1 *Acinetobacter baumannii*, 1 coagulase-negative *Staphylococcus* species, and 1 *Streptococcus mitis* and *Streptococcus oralis* case. In all cases excluding the coagulase-negative *Staphylococcus* species case, which was not analyzed because of a lack of species-level identification, the estimated biomass (ng/mL peritoneal effluent supernatant) of the culture-confirmed organism in the peritoneal effluent within 2 days of diagnosis was higher than the median biomass of the same species in specimens that were confirmed to be culture-negative in the no-peritonitis group (Fig. 2A). The cfDNA abundances of common bacterial species are also graphically represented by culture-confirmed peritonitis and culture-negative peritonitis cases (Fig. 2B; Table S1). Correlational analyses were also performed between the host cfDNA and white blood

cell concentration, and there was a significant positive correlation of Spearman's ρ of 0.78 ($P < 0.001$; Fig S2). We also analyzed the antibiotic-resistant genes present in the 11 culture-confirmed peritonitis cases and found a number of antibiotic-resistant genes within each of the specimens (Fig S3). However, given the low sample size of antimicrobial susceptibility testing patterns (7 of 11 were available), we were not able to make correlations between cfDNA and antimicrobial susceptibility patterns.

A major challenge for cfDNA metagenomic sequencing is its relatively poor specificity due to alignment noise and environmental contamination.[15] To overcome this limitation, we recently developed LBBC, a metagenomics noise-filtering tool informed by the uniformity of the coverage of microbial genomes and the batch variation in the absolute abundance of microbial cfDNA.[4] We applied LBBC to all 68 data sets and 18 replicates and aggregated and visualized the data at the species level (Fig 3A). Noise filtering by LBBC improved the specificity of the metagenomics analysis without greatly reducing the sensitivity. The exceptions for identification included: a case of *S oralis* and *S mitis*, a case of *A baumannii*, a case of *E coli*, a case of coagulase-negative *Staphylococcus* species, and a case of *S epidermidis* for which metagenomic hits were filtered out by LBBC.

Importantly, in the *K pneumoniae* and *S haemolyticus* cases, cfDNA sequencing detected the same organisms in culture-confirmed peritonitis even after antibiotic therapy and even though the conventional cultures were negative after antibiotic therapy but positive before antibody therapy. Our data suggest that cfDNA within 2 days of peritonitis can still be diagnostic of peritonitis despite recent antibiotic therapy and the conventional culture being negative in the setting of antibiotic therapy.

In the peritonitis group, there were 4 patients with a total of 5 episodes of culture-negative peritonitis who were treated as if they had clinical peritonitis, and 1 culture-confirmed PD patient who subsequently developed culture-negative peritonitis. In 1 patient with culture-negative peritonitis, the top 2 microbial species identified were *Enterococcus faecalis* and *Parabacteroides distasonis*. This patient did not improve after antibiotic therapy for

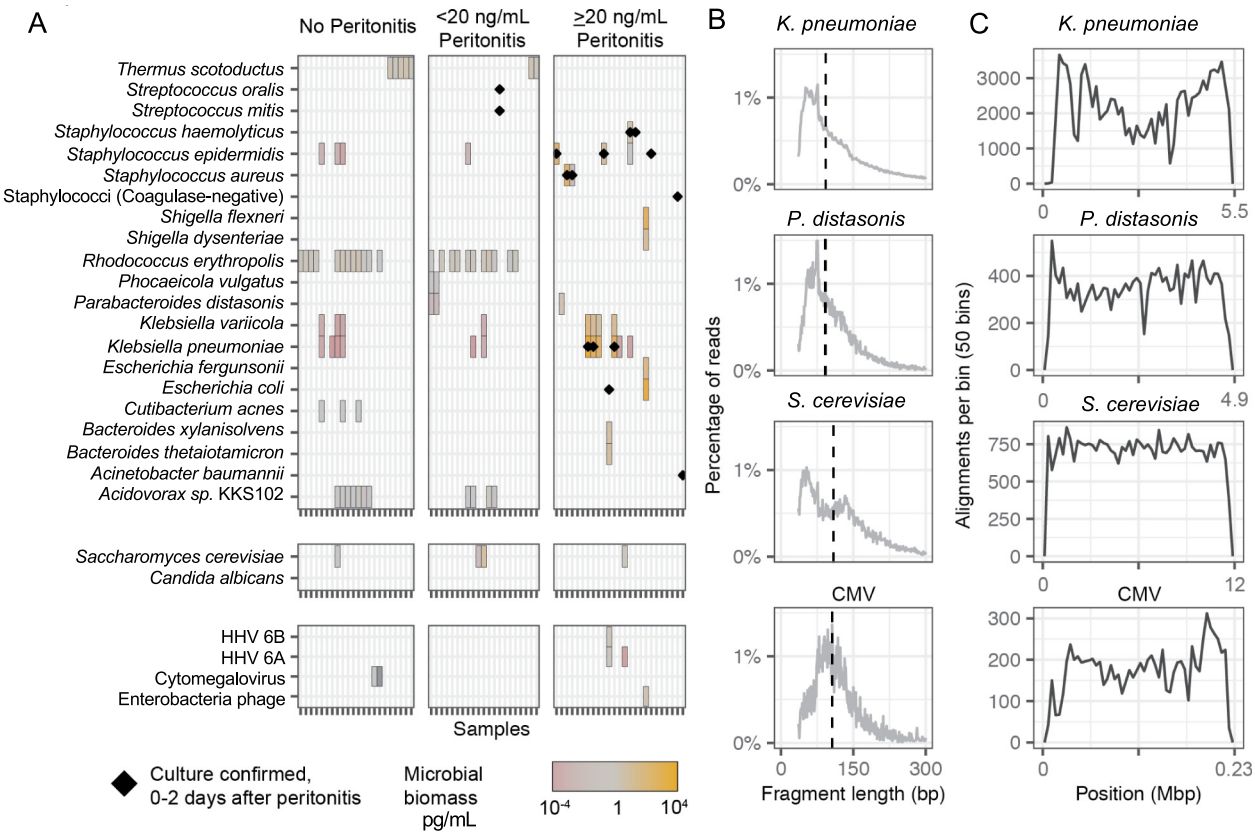

Figure 3. Metagenomic sequencing of cell-free DNA from peritoneal effluent reveals presence of pathogens in the peritoneum. (A) A biomass of various microbial organisms detected in samples across the cohort is shown. (B) In select cases, the fragment length histogram of reads aligning to *Klebsiella pneumoniae* (culture confirmed in peritoneal effluent), *Parabacteroides distasonis* (culture confirmed from gallbladder), *Saccharomyces cerevisiae* (not recovered in culture), and cytomegalovirus (no culture/polymerase chain reaction) are shown. Vertical lines represent median value in the range of 25 to 300 bp. (C) For organisms in panel B, sequencing coverage across respective microbial genomes is shown as the number of read alignments per bin (bin size variable; 50 total bins per plot). Abbreviations: CMV, cytomegalovirus; HHV, human herpesvirus.

presumed peritonitis and eventually had a cholecystotomy for presumed cholecystitis. The cultures from the cholecystotomy drain grew *E faecalis* and *P distasonis*. Given the ability of *E faecalis* and *P distasonis* to grow efficiently on conventional media, it is likely that the patient truly had culture-negative peritonitis and that the detected cfDNA diffused from the gallbladder. Our limited data suggest that peritoneal effluent cfDNA can diagnose infections beyond peritonitis. In another PD patient with culture-negative peritonitis, while microbial cfDNA was not initially detected above background, an elevated amount of *E coli* was detected 11 days after presentation and after antibiotic treatment. In one other patient, low levels of *P distasonis* and *Proteus vulgaris* were detected, and in the 2 other patients, there was no identified pathogen above background.

Identification of Viral and Eukaryotic Pathogenic cfDNA in Peritoneal Effluent

We further detected cfDNA derived from human viruses and bacteriophages in samples from patients with and without peritonitis (Fig 3A). In the case of the culture-negative peritonitis in which cfDNA sequencing revealed the presence of *E coli*, we detected cfDNA from an enterobacteria phage cfDNA (3.1 pg/mL). In another sample from a patient with diagnosed *E coli* peritonitis, we found a high burden of human herpesvirus (HHV-6, >2 pg/mL). In another PD patient who presented for a routine visit and no evidence of clinical peritonitis, we observed a high burden of human cytomegalovirus (1.1 pg/mL). We also observed cfDNA derived from fungi in a specimen from a patient with a history of recent peritonitis. Interestingly, we observed a high level of *Saccharomyces cerevisiae* cfDNA at 15 days after detection of *Klebsiella pneumoniae* peritonitis. The patient was treated with fluconazole prophylactically after the peritonitis episode, but eventually, the patient had her catheter removed due to recurrent peritonitis and cyst infections. To further confirm the identification of *S cerevisiae*, we compared BLAST reads in a paired-end context (see Methods). We found that *S cerevisiae* genome fragments were distributed, as expected, with a median fragment length exceeding 90 bp and a local peak at approximately 144 bp, representing the length of DNA in the yeast nucleosome (Fig 3B).[16] We compared this to cfDNA aligned to *K pneumoniae*, *P distasonis*, and cytomegalovirus, and found high median length values for all cases (Fig 3B).

DISCUSSION

We report the first in-depth analysis of the properties of cfDNA in peritoneal effluent and a study of the utility of metagenomic sequencing of cfDNA in peritoneal effluent for screening infections within the peritoneal cavity. We found that peritoneal effluent cfDNA sequencing can not only detect pathogens consistent with culture-confirmed peritonitis in the majority of cases, but also detect bacterial, viral, and fungal organisms in culture-negative peritonitis and after antibiotic therapy, demonstrating the power of cfDNA-based diagnostics beyond conventional culture.

Positive body fluid cultures are among the criteria for diagnosing peritonitis by the ISPD 2016 guidelines.[7] While culture can diagnose infection in a majority of cases, cultures are negative in approximately 20% of PD patients, leading to patients being presumptively treated for culture-positive peritonitis.[1] In these particularly difficult cases, metagenomic sequencing of cfDNA may have several advantages over conventional culture. First, antibiotics that can diffuse into the peritoneum or are directly instilled into the peritoneum can interfere with the culturing process. As shown in our study, we detected cfDNA from the same pathogen in culture-confirmed cases within 2 days of antibiotic therapy even though corresponding peritoneal effluent cultures were negative. Second, the inflammatory process in the peritoneum can be related to processes beyond infection of the peritoneal effluent. We have presented a case in which the nidus of infection was the gallbladder in a PD patient who was treated for presumed peritonitis and the bacterial cfDNA in the gallbladder likely diffused into the peritoneal cavity, and both suspected bacterial pathogens were identified using cfDNA sequencing of the peritoneal effluent. It is conceivable that PD patients with culture-negative peritonitis have abdominal infections beyond peritonitis and that cfDNA sequencing may identify suspected pathogens. Given the expected low biomass of the peritoneal effluent, we have implemented a filtering process that eliminates background contamination. While this process eliminates many organisms that are likely contaminants in the processing steps, it does filter out some of the culture-confirmed positive organisms. A larger study to maximize the filtering process for false positives but minimize the elimination of true positives is needed.

Peritoneal effluent cfDNA sequencing further identified viral and fungal DNA in peritoneal effluent that was otherwise undetected in PD patients. Cytomegalovirus (HHV-5) was identified in a PD patient who presented to the clinic for routine follow-up and Roseolovirus (HHV-6) was identified in a patient with concurrent *E coli* peritonitis. In a patient who received treatment for *K pneumoniae* peritonitis and antifungal prophylaxis with fluconazole, metagenomic cfDNA sequencing identified *S cerevisiae*. In this case, we attempted to culture the *S cerevisiae* from a stored peritoneal effluent specimen using conventional fungal culture, but we were unable to recover *S cerevisiae* in culture. The detection of these specific viral and fungal elements, however, raises the question of whether these pathogens are the cause of disease. In the case of a PD patient with detectable cytomegalovirus cfDNA, the patient was not symptomatic, and so our assay might have detected the latent virus. For the PD patient with detectable *S cerevisiae*, it is possible that the *S cerevisiae* cfDNA was diffusing from the gut. It is also possible that diffusion of cfDNA occurs from the plasma to peritoneal fluid because of increased peritoneal permeability and is not present in the abdominal

cavity. While peritoneal effluent cfDNA sequencing may be able to detect microbial cfDNA at high resolution, further studies that concurrently examine plasma cfDNA are needed to better understand whether the microbes detected by this technology are causing the diseased state.

Next-generation sequencing of peritoneal effluent cfDNA has the potential to aid in the diagnosis of challenging cases, such as culture-negative peritonitis or recurrent peritonitis, and to predict relapse of peritonitis. When compared to conventional culture, the widespread adoption of such a technique may be difficult given the complexity of analyses, especially in resource-limited settings. However, many advances in next-generation sequencing technology have led to decreased costs, decreased turnaround times, and increased portability. Nanopore sequencers, for example, can be purchased for approximately $1,000 US dollars (in 2021) and can provide real-time sequencing data with a small-footprint, portable device. Indeed, a recent study by Gu et al[17] reported on using Nanopore sequencing for body fluids, and reported a median 50-minute sequencing time and a 6-hour sample-to-answer time.

In summary, our study identifies peritoneal effluent cfDNA sequencing as a novel method to characterize both culture-positive and culture-negative peritonitis in PD patients. Further studies are needed to validate the utility of peritoneal effluent cfDNA sequencing in challenging cases of culture-negative peritonitis and recurrent peritonitis.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1: Human cfDNA and microbial cfDNA in peritonitis cases over time.

Figure S2: Human cfDNA biomass correlates with peritoneal fluid white blood cell.

Figure S3: Antibiotic resistant profiles in peritonitis cases.

Item S1: Potential adapters present in sample.

Table S1: cfDNA species biomass are represented in the 11 culture-confirmed peritonitis cases and the 10 no peritonitis cases confirmed by culture.

ARTICLE INFORMATION

Authors' Full Names and Academic Degrees: Philip Burnham, PhD, Fanny Chen, BS, Alexandre P. Cheng, PhD, Vesh Srivatana, MD, Lisa T. Zhang, MD, Emmanuel Edusei, BS, Shady Albakry, BA, Brittany Botticelli, BA, Xunxi Guo, MD, Amanda Renaghan, MD, Jeffrey Silberzweig, MD, Darshana M. Dadhania, MD, Joan S. Lenz, PhD, Michael Heyang, BS, Iliyan D. Iliev, PhD, Joshua A. Hayden, PhD, Lars F. Westblade, PhD, Iwijn De Vlaminck, PhD, and John R. Lee, MD.

Authors' Affiliations: Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY (PB, FC, APC, JSL, MH, IDV); The Rogosin Institute, New York, NY (VS, JS); Division of Nephrology and Hypertension, Department of Medicine, Weill Cornell Medicine, New York, NY (LTZ, EE, SA, BB, XG, AR, DMD, JRL); Department of Transplantation Medicine, New York-Presbyterian Hospital–Weill Cornell Medical Center, New York, NY (DMD, JRL); Division of Gastroenterology and Hepatology, Department of Medicine, Weill Cornell Medicine, New York, NY (IDI); Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY (JAH, LFW); Division of Infectious Diseases, Department of Medicine, Weill Cornell Medicine, New York, NY (LFW); Department of Bioengineering, University of Pennsylvania, Philadelphia, PA (PB, current affiliation); and Department of Chemistry, Norton Healthcare, Louisville, KY (JAH, current affiliation).

Address for Correspondence: Iwijn De Vlaminck, Meinig School of Biomedical Engineering, Cornell University, Weill Hall Room 301, Ithaca, NY, 14853. Email: vlaminck@cornell.edu or John R. Lee, Division of Nephrology and Hypertension, Department of Medicine, Weill Cornell Medicine, 525 E. 68th Street Box 3, New York, NY, 10065. Email: jrl2002@med.cornell.edu

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