Title

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Journal

The Journal of molecular diagnostics : JMD, 21(5)

ISSN

1525-1578

Authors

Culbreath, Karissa
Melanson, Suzanne
Gale, James
et al.

Publication Date

2019-09-01

DOI

10.1016/j.jmoldx.2019.05.002

Peer reviewed

The Journal of Molecular Diagnostics, Vol. 21, No. 5, September 2019

the Journal of Molecular Diagnostics
jmd.amjpathol.org

Validation and Retrospective Clinical Evaluation of a Quantitative 16S rRNA Gene Metagenomic Sequencing Assay for Bacterial Pathogen Detection in Body Fluids

Karissa Culbreath,*† Suzanne Melanson,† James Gale,† Justin Baker,† Fan Li,‡ Oystein Saebo,§ Oyvind Kommedal,¶ Deisy Contreras,‖ Omai B. Garner,‖ and Shangxin Yang*‖

From the Department of Pathlogy, University of New Mexico Health Sciences Center, Albuquerque, New Mexico; TriCore Reference Laboratories,† Albuquerque, New Mexico; the Department of Pediatrics‡ and the Clinical Microbiology Laboratory,‖ Department of Pathology and Laboratory Medicine, University of California Los Angeles, Los Angeles, California; Pathogenomix Inc.,§ Santa Cruz, California; and the Department of Microbiology,¶ Haukeland University Hospital, Bergen, Norway*

Accepted for publication May 24, 2019.

Address correspondence to Shangxin Yang, Ph.D., UCLA Clinical Microbiology Laboratory, Brentwood Annex, 11633 San Vicente Blvd, Los Angeles, CA 90049. E-mail: shangxinyang@mednet.ucla.edu.

Next-generation sequencing—based 16S rRNA gene metagenomic sequencing (16S MG) technology has tremendous potential for improving diagnosis of bacterial infections given its quantitative capability and culture-independent approach. We validated and used a quantitative 16S MG assay to identify and quantify bacterial species in clinical samples from a wide spectrum of infections, including meningitis, septic arthritis, brain abscess, intra-abdominal abscess, soft tissue abscess, and pneumonia. Twenty clinical samples were tested, and 16S MG identified a total of 34 species, compared with 22 species and three descriptive findings identified by culture. 16S MG results matched culture results in 75% (15/20) of the samples but detected at least one more species in five samples, including one culture-negative cerebrospinal fluid sample that was found to contain *Streptococcus intermedius*. Shotgun metagenomic sequencing verified the presence of all additional species. The 16S MG assay is highly sensitive, with a limit of detection of 10 to 100 colony-forming units/mL. Other performance characteristics, including linearity, precision, and specificity, all met the requirements for a clinical test. This assay showed the advantages of accurate identification and quantification of bacteria in culture-negative and polymicrobial infections for which conventional microbiology methods are limited. It also showed promises to serve unmet clinical needs for solving difficult infectious diseases cases. *(J Mol Diagn 2019, 21: 913—923; https://doi.org/10.1016/j.jmoldx.2019.05.002)*

Currently, there are many challenges and unmet clinical needs in the diagnosis of infectious diseases. Conventional diagnosis relies heavily on culture-dependent microbiology, which has limitations, including the following: i) long incubation time; ii) polymicrobial and complex infections; iii) antimicrobial treatment before culture; and iv) lack of sensitivity. To overcome these limitations, culture-independent molecular tests have gained large-scale adoption in modern-day clinical microbiology, as demonstrated by the wide use of nucleic acid amplification technologies for pathogen detection. However, these tests are still limited to a narrow spectrum of the most common pathogens.

The next-generation sequencing (NGS)—based metagenomic approaches comprise two distinct methods:

Supported by a University of New Mexico Department of Pathology pilot grant (K.C.) and by Pathogenomix Inc. (Santa Cruz, CA).

Disclosures: O.K. has consulted as a medical adviser for Pathogenomix Inc. and has contributed to the development of the RipSeq software. He is also a minor shareholder in Pathogenomix Inc. O.S. has contributed to the development of the RipSeq software and is a shareholder in Pathogenomix Inc.

https://doi.org/10.1016/j.jmoldx.2019.05.002

targeted approach, such as 16S rRNA gene sequencing[1,2]; and metagenomic shotgun sequencing.[3] These two methods have recently been used for infectious diseases diagnosis because of their much broader coverage of pathogen detection compared with nucleic acid amplification technologies. Both have shown potential for solving the difficult infectious diseases cases that are insidious, complex, or unexpected, for which both culture and nucleic acid amplification technology results are often negative or not helpful.[4–6]

Shotgun metagenomics is advantageous in detecting bacteria, parasites, viruses, fungi, and resistance markers. However, it is limited by several factors, including the following: i) requirement for a much larger amount of sequencing data because of interference of human DNA, ii) higher cost per test, iii) lower throughput per test run, and iv) relatively lower sensitivity.[7] On the other hand, 16S rRNA gene metagenomic sequencing (16S MG) overcomes many of the obstacles of shotgun metagenomics, thereby making it a more cost-effective and higher-throughput option as a practical clinical test for diagnosing bacterial infections. Recent studies have also highlighted the ability of 16S MG to accurately quantify bacterial abundances in complex population mixtures.[8] In this study, we developed and validated a quantitative 16S MG test and compared its results with conventional culture results in 20 body fluid and abscess samples. We demonstrate that accurate speciation of mixed bacteria and precise quantification of bacterial abundance may help clinicians better understand the extent and nature of the infection, which could lead to better treatment decisions and reduced health care costs.

Materials and Methods

Clinical Samples

This study was approved by the University of New Mexico (Albuquerque, NM) Human Research Protection Committee. Residual clinical samples collected from 2013 to 2016 and stored at −20°C were retrieved and tested retrospectively (Table 1). To maximize the data that can be generated for technical performance validation, most samples included in this study were culture positive. However, because of the agnostic nature of the 16S MG assay, each culture-positive sample is also evaluated as a negative sample for all bacteria that did not grow by culture. The clinical information and all related laboratory results were also reviewed.

16S MG Assay

A total of 400 μL of sample was mixed with 400 μL bacterial lysis buffer in a bead tube provided in the SeptiFast Lys kit (Roche Molecular Diagnostics, Pleasanton, CA). The tube was then placed onto a Disruptor Genie (Scientific Industries, Bohemia, NY) and vortex mixed twice for 2 minutes at maximum speed. The tube was then centrifuged at 10,000 × *g* for 3 minutes, and 200 μL supernatant was transferred from the tube for DNA extraction on the MagNA Pure LC 2.0 automatic extraction system (Roche Molecular Diagnostics, Pleasanton, CA). The elution volume was 50 μL. The qubit system (Thermo Fisher Scientific, Waltham, MA) was used to quantify the DNA concentration, which ranged from 2 to 2666 ng/μL (median = 164 ng/μL). The V1 to V2 region of the 16S rRNA gene was amplified by PCR using the primer sets and conditions described by Kommedal et al.[9] A positive (cultured bacteria mix used for linearity validation or a culture-positive clinical sample used in precision validation) and a negative (molecular-grade water) control were included in each run. The PCR products were visualized and quantified by using BioAnalyzer 2100 (Agilent, Santa Clara, CA), and 100 ng of each was then processed to prepare libraries using the Ion Plus Fragment library kit (Thermo Fisher Scientific) with Agencourt AMPure XP kit (Beckman Coulter, Danvers, MA) for sample purification following the manufacturer's protocol. In the cases of negative controls and samples with low biomass, due to low concentration of the PCR products (Supplemental Figure S1), the undiluted maximum volume was used for the downstream library preparation. A sample of each library (10 μL) was pooled (four to six samples plus the two controls) and diluted to 12 pmol/L, amplified, and enriched using the One Touch 2 and the OT2 HiQ View 400 kit (Thermo Fisher Scientific) following the manufacturer's protocol. The libraries were loaded onto an Ion Torrent 318 chip and then sequenced on the Ion Torrent PGM (both from Thermo Fisher Scientific). Approximate sequencing run time was 7 hours 20 minutes. An average of 1,054,809 (range, 537,936 to 1,975,045) reads were acquired per clinical sample.

Shotgun Metagenomics

The same extracted DNA was used for shotgun metagenomic sequencing. Briefly, 1 μg of total DNA was fragmented using the Ion Shear Plus Reagents kit (Thermo Fisher Scientific). Human DNA depletion was not performed. The libraries were prepared using Thermo Fisher Scientific reagents, as described in the IonXpress Plus gDNA Fragment Library preparation protocol. The libraries were loaded onto the Ion Torrent 318 chips (1 sample per chip) and then sequenced using the PGM sequencer. An average of 5.2 million (range, 4.8 to 5.7 million) reads were acquired per clinical sample.

Validation of Linearity

Two sets of linearity panels were prepared by mixing laboratory cultured and quantified (by turbidity in McFarland) *Staphylococcus aureus* and *Enterococcus faecalis* (Gram-positive panel) or *Escherichia coli* and *Klebsiella pneumoniae* (Gram-negative panel) at ratios of 1:10, 1:3, 1:1, 3:1, and 10:1 in water, which generated a range of bacterial

Table 1 Samples Tested by 16S MG in This Study

Sample type	No. of samples
Abscess, breast	1
Abscess, cerebellar	1
Abscess, pharyngeal	2
Abscess, shoulder	1
Bile	3
Kidney fluid	2
Bronchial alveolar lavage	1
CSF	4
Pleural fluid	1
Synovial fluid	4
Negative control (molecular-grade water)	10
Spiked samples in water for linearity validation	10
Spiked samples in water for LOD validation	12
Spiked samples in body fluids for LOD and IC validation	5
Total	57

CSF, cerebrospinal fluid; IC, internal control; LOD, limit of detection.

relative abundances between 9% and 91%. Each five-sample panel was then tested by 16S MG in the same manner as the clinical samples. The measured log ratios of the relative abundance of the mixed bacteria were plotted against expected log ratios, and a linearity curve was generated using Microsoft Excel (Microsoft Corp., Redmond, WA).

Validation of Limit of Detection and Internal Control

Six bacterial species, including *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, *S. aureus*, *E. faecalis*, and *Clostridium sordellii*, were each spiked into water and diluted to generate 12 samples at the concentrations of 100 and 10 colony-forming units (CFUs)/mL. In addition, seven bacterial species, including *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *E. faecalis*, *Clostridium perfringens*, and a *Bacteroides* species, were mixed in water to generate a pool with the concentration of 1000 CFUs/mL for each species. *Marinobacter aquaeolei* (700491; ATCC, Manassas, VA), which served as an internal control, was also spiked in this pool to reach a concentration of 5000 CFUs/mL. This pool was then spiked into five different body fluids (residual culture–negative clinical samples), including bronchoalveolar lavage (BAL), cerebrospinal fluid (CSF), peritoneal fluid, pleural fluid, and synovial fluid, at a 1:10 ratio, resulting in a final concentration of 100 CFUs/mL for all of the seven clinically relevant bacteria and 500 CFUs/mL for the internal control. All the 12 samples in water and the five samples in body fluids were tested by the 16S MG in the same manner as the clinical samples.

Data Analysis

Sequence data were analyzed using a commercial cloud-based software platform (RipSeq; Pathogenomix, Santa Cruz, CA). The demultiplexed FASTQ files were uploaded to the cloud, and the results were downloaded in PDF format. The main steps in the bioinformatics pipeline include sequence trimming and filtering, clustering, and species identification based on the representative sequence of each cluster. The database used included a curated 16S database combined with National Center for Biotechnology Information RefSeq. In this study, the software has been optimized to set the following thresholds to reduce background: i) remove reads with <200 bp in length; ii) remove species with <200 reads aligned; and iii) remove species with <0.1% relative abundance. For shotgun metagenomics data analysis, taxonomic classification was performed using Kraken version 1.0[10] against a database of all complete bacterial, fungal, archael, and viral genomes from National Center for Biotechnology Information RefSeq. Reads mapped to each species by Kraken were also pulled out and *de novo* assembled into contigs using Geneious version 8.1 (Biomatters, Auckland, New Zealand). The contigs were then blasted against the National Center for Biotechnology Information database to confirm the species identification.

Results

Performance Characteristics of the 16S MG Assay

Accuracy

Twenty clinical samples plus 10 negative controls and 10 linearity panel samples used as positive controls were tested (Table 1). In 75% of the clinical samples (15/20), the 16S MG results agreed with the culture results. However, in five clinical samples, 16S MG identified at least one more species that was not identified by culture (Tables 2 and 3). 16S MG identified a total of 34 species compared with only 26 species by culture (Table 3). All eight additional bacterial species were verified by shotgun metagenomic sequencing (Table 2). The most significant discrepant result was a culture-negative CSF sample in which *Streptococcus intermedius* (100% abundance) was detected by the 16S MG and further confirmed by shotgun metagenomics, with 4846 reads aligned to the *S. intermedius* reference genome CP003858.1 (pairwise identity = 97.7%). The only minor error was found in a BAL (sample 18) that grew mixed upper respiratory flora and the 16S MG detected *Corynebacterium propinquum*, which was actually a mixture of multiple closely related *Corynebacterium* species, including *C. propinquum* shown by the shotgun metagenomic sequencing (Table 2). This error indicates an insufficient resolution in the V1 to V2 region of the 16S rRNA gene to differentiate mixed *Corynebacterium* species.

Linearity and Reportable Range

To assess the quantitative capability of this assay for relative abundance, two linearity panels were generated by artificially mixing two laboratory-cultured bacteria (*S. aureus* and *E. faecalis* or *E. coli* and *K. pneumoniae*) at a set of ratios so that the relative abundance ranged from 9% (1:10

Table 2 Result Summary of the Clinical Samples

ID no.	Clinical presentations	Sample type	16S MG results (RipSeq)	
			Total reads	Species ID
1	Kidney stones	Kidney fluid	1,033,639	*Escherichia coli*
2	Empyema	Pleural fluid	1,243,377	*Streptococcus pyogenes*
3*	Cholecystitis	Gallbladder fluid	939,122	*Klebsiella pneumoniae* ***Clostridium perfringens***
4	Humerus fracture	Shoulder abscess	1,164,105	*Staphylococcus aureus*
5	Meningitis	CSF	812,031	*K, pneumoniae*
6	Arthritis	Synovial fluid	971,027	*S. aureus*
7	Renal abscess	Kidney fluid	1,021,861	*E. coli*
8	Meningitis	CSF	1,182,719	*Serratia marcescens*
9	Pharyngeal abscess	Pharyngeal abscess	1,477,595	*S. pyogenes*
10	Pharyngeal abscess	Pharyngeal abscess	1,550,837	*S. pyogenes*
11	Meningitis	CSF	613,030	*S. marcescens*
12	Breast abscess	Breast abscess	1,276,750	*S. aureus*
13	Arthritis	Synovial fluid	1,007,276	*S. aureus*
14	Arthritis	Synovial fluid	1,975,045	*S. pyogenes*
15*	Perihepatic abscess	Bile	1,078,158	*Citrobacter freundii* *K. pneumoniae* ***Klebsiella oxytoca*** ***Clostridium perfringens***
16*	Brain abscess	Brain abscess	537,936	***Fusobacterium nucleatum*** *Streptococcus intermedius* ***Capnocytophaga* spp.** *Aggregatibacter aphrophilus*
17	Arthritis	Synovial fluid	904,899	*S. aureus*
18*	Pneumonia	BAL	537,936	*Pseudomonas aeruginosa* ***Corynebacterium propinquum*** *Streptococcus pneumoniae*
19*	Meningitis	CSF	955,154	***S. intermedius***
20*	Intra-abdominal abscess	Bile	813,691	***K. pneumoniae*** ***Citrobacter freundii*** *P. aeruginosa* ***Fusobacterium periodonticum*** ***Enterococcus faecalis*** ***Hemophilus parainfluenzae***

(*table continues*)

*Only the six samples with discordant species detection/identification results (in bold) between 16S MG and culture were further tested by shotgun MG.

BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; ID, identification; NA, not available.

mixing ratio) to 91% (10:1 mixing ratio) for each species. 16S MG testing of these samples showed linear correlation between the measured log ratios of the relative abundances and the expected log ratios (Pearson correlation coefficient $R^2 = 0.99$) (Figure 1). These results indicate that the 16S MG assay is able to quantify the relative abundance of bacteria within a range of 9% to >90%.

Precision

To validate the precision of the assay, the acceptable criteria were first defined as 95% CI < means ± 15% for the high-abundance species (>50%); 95% CI < means ± 10% for medium-abundance species (10% to 49%); and 95% CI < means ± 5% for low-abundance species (<10%). A BAL sample that happened to have three species at different abundance levels (high, medium, and low) for six times in five separate runs performed on different days by different technologists was then repeatedly tested. In the second run, the sample was tested twice to also assess the intra-assay precision. The 16S MG assay not only reproduced identical results but also met the precision criteria with the 95% CI of means ± 9.1% for the high-abundance *P. aeruginosa*, the 95% CI of means ± 6.8% for the medium-abundance *C. propinquum*, and the 95% CI of means ± 1.9% for the low-abundance *S. pneumonia* (Table 4).

Analytical Sensitivity

When bacteria were spiked into water, the limit of detection (LOD) was determined to be <100 CFUs/mL. All the six species were detected at 100 CFUs/mL, and three species

Table 2 (*continued*)

16S MG results (RipSeq)			Shotgun MG results (Kraken)	
Matched reads	Abundance, %	Culture results	Species ID	Matched reads
606,517	100.0	*E. coli*	NA	NA
804,548	99.9	*S. pyogenes*	NA	NA
469,405	87.9	*K. pneumoniae*	*K. pneumoniae*	53
62,881	**11.8**	**Not found**	***C. perfringens***	**137**
642,180	100.0	*S. aureus*	NA	NA
465,644	98.8	*K. pneumoniae*	NA	NA
408,059	100.0	*S. aureus*	NA	NA
606,557	100.0	*E. coli*	NA	NA
824,926	100.0	*S. marcescens*	NA	NA
695,051	98.0	*S. pyogenes*	NA	NA
648,056	98.1	*S. pyogenes*	NA	NA
261,298	91.1	*S. marcescens*	NA	NA
348,451	97.3	*S. aureus*	NA	NA
536,055	99.9	*S. aureus*	NA	NA
393,383	92.2	*S. pyogenes*	NA	NA
256,116	50.1	*C. freundii*	*C. freundii*	8162
154,875	30.3	*K. pneumoniae*	*K. pneumoniae*	6998
77,322	**15.1**	**Not found**	***K. oxytoca***	**3951**
14,864	**2.9**	**Not found**	***C. perfringens***	**12**
118,521	**42.7**	**Mixed anaerobic flora**	***F. nucleatum***	**273**
83,891	30.2	*S. intermedius*	*S. intermedius*	5984
54,499	**19.6**	**Not found**	***Capnocytophaga* spp.**	**153**
18,641	6.7	*A. aphrophilus*	*A. aphrophilus*	439
351,142	95.4	*S. aureus*	NA	NA
538,480	71.9	*P. aeruginosa*	*P. aeruginosa*	183,769
191,891	**25.6**	**Mixed upper respiratory flora**	***Corynebacterium* spp. (mixed)**	**3843**
10,708	1.4	*S. pneumoniae*	*S. pneumoniae*	1739
494,722	**100.0**	**Not found**	***S. intermedius***	**4846**
243,085	**61.7**	**Mixed Gram-negative rods**	***K. pneumoniae***	**8576**
50,561	**12.8**	**Mixed Gram-negative rods**	***C. freundii***	**14,266**
36,325	9.2	*P. aeruginosa*	*P. aeruginosa*	5112
13,575	**3.4**	**Not found**	***F. periodonticum***	**6**
11,397	**2.9**	**Not found**	***E. faecalis***	**12,412**
4570	**1.2**	**Not found**	***H. parainfluenzae***	**826**

were detected at 10 CFUs/mL (Table 5). More important, at the low concentrations when the intensity of the 16S PCR amplicon from the spiked samples was invisible and shown to be even less than that of the negative control (Supplemental Figure S1), the 16S MG could still accurately detect the bacteria with high abundance (median, 37.8%; minimum, 1.9%; and maximum, 51.4%). When bacteria were spiked into body fluids, the LOD varied, depending on the type of body fluid and the number of total reads acquired. When the total reads exceeded 1.5 million, all of the seven spiked bacteria were detected at 100 CFUs/mL in both the peritoneal fluid and pleural fluid; when the total reads decreased to 1.2 million, only four spiked bacteria were detected at 100 CFUs/mL in the BAL; and when the total reads decreased to <0.5 million, only two spiked bacteria were detected in the CSF but none were detected in the synovial fluid (Table 6). These results suggested 1.5 million total reads per sample is optimal for this test to achieve its maximal sensitivity. In the BAL, bacterial DNA from the normal oral flora, including *Prevotella* species, *Actinomyces* species, *Rothia* species, *Lachnoanaerobaculum* species, and *Streptococcus* species, was also detected with a combined abundance of >20%; and in the synovial fluid, 40,359 reads (54.4% abundance) of *Propionibacterium acnes* were detected. The preexisting bacterial DNA in the fluids may interfere with the spiked bacterial DNA and lead to a lowered analytical sensitivity in these two samples.

Internal Control

A bacterium that lives in the ocean, *Marinobacter aquaeolei*,[11] was selected as the internal control because of two

Table 3 Overall Result Comparison between 16S MG and Culture on the Clinical Samples

Result comparison by sample							Result comparison by species				
		Culture							Culture		
		Negative	One species	Two species	Three species	Total			Detected	Not detected	Total
16S MG	Negative	**0**	0	0		0	16S MG	Detected	24	8*	34
	One species	1	**14**	0		15					
	Two species	0	1	**0**		1		Not detected	0	0	0
	Three species	0	0	0	**1**	1					
	Greater than three species	0	0	2	1	3		Total	24	8	34
	Total	1	15	2	2	20					

*All of the eight additional species were detected by shotgun metagenomic sequencing. Bold text indicates the number of samples with concordant results between culture and 16S MG.

advantages: it is a marine bacterium that has never been reported to cause human infection and is highly unlikely to cause human infection; and it is not present in the laboratory environment or in the reagents as a contaminant. In all of the five body fluids spiked with the internal control at a final concentration of 500 CFUs/mL, it was readily detected, with >7000 reads, and did not show to interfere with the LOD in the peritoneal fluid and pleural fluid, even when the abundance was as high as 20.1% (Table 6). These results demonstrated that this internal control could be reliably used in the test system.

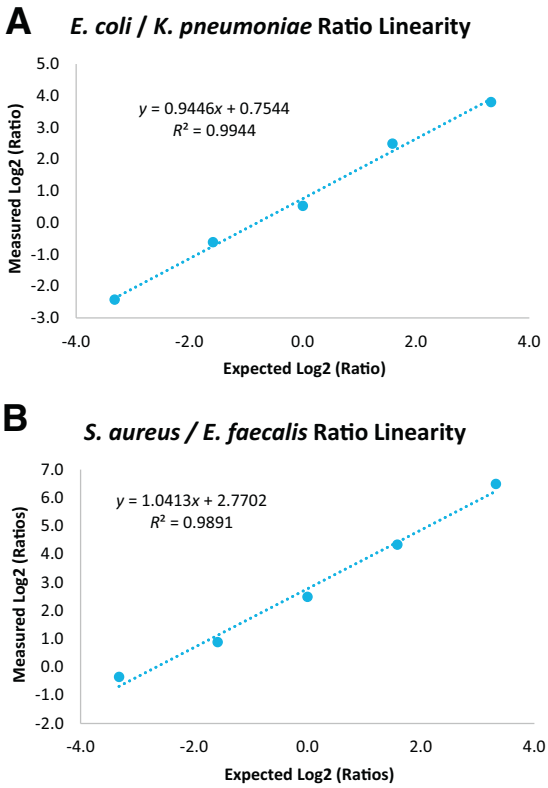

Figure 1 Plots of the logarithmic value (Log2) of the ratios of the measured relative abundance (*y* axis) against the Log2 of the ratios of the expected relative abundance (*x* axis) in a panel of five samples spiked with two different bacteria at mixing ratios of 1:10, 1:3, 1:1, 3:1, and 10:1. The **blue dotted line** is the linear trendline, and the **larger blue dots** represent the ratios of the mixed bacteria. **A:** Results of the Gram-negative panel (*Escherichia coli* and *Klebsiella pneumoniae*). **B:** Results of the Gram-positive panel (*Staphylococcus aureus* and *Enterococcus faecalis*).

Analytical Specificity

Ten negative controls, as well as 22 spiked samples in molecular-grade water, were tested in the same manner as the clinical samples; and a list of laboratory contaminants (defined as bacteria that were not spiked in and not supposed to be detected) was identified (Supplemental Table S1). Not all these contaminants showed up in each test. The number of contaminant species in each sample varied from zero to six species (median = 1, mean = 0.87). *Propionibacterium acnes* was the most common laboratory contaminant; however, its abundance was all <10% in the clinical samples and spiked body fluids, except for a synovial fluid sample used for LOD validation, in which 54.4% of *P. acnes* was detected, as described in *Analytical Sensitivity*. This accidental finding of highly abundant *P. acnes* in a randomly picked culture-negative synovial fluid might represent a true joint infection by *P. acnes*. To avoid reporting out any false-positive results due to laboratory contamination, species that were detected in the negative control of the run were filtered out from the results of clinical samples. Furthermore, a higher cutoff (20% abundance), determined on the basis of the observation that all laboratory contaminants, except *P. acnes*, are <20% abundant in the clinical samples or spiked body fluids, was used to filter out contaminants. If a species on the laboratory contaminant list, except *P. acnes*, was detected with an abundance >20% and reported, a disclaimer would be attached stating "the detection of this species might be due to environment contamination, interpret the results with caution." For *P. acnes*, abundance >20% may represent a true infection and, therefore, will be reported out, especially in the case of joint infections. By using these criteria, 100% specificity was achieved for the clinical samples and the spiked body fluid samples.

Table 4 Validation of Precision by Repeated Testing of a BAL Sample

Bacteria	First run	Second run no. 1*	Second run no. 2*	Third run	Fourth run	Fifth run	Mean	SD	95% CI
Pseudomonas aeruginosa	74.7	60.6	61.6	85.8	82.2	84.5	74.9	11.4	±9.1
Corynebacterium propinquum	23.6	32.6	32.1	13.8	17.1	14.7	22.3	8.5	±6.8
Streptococcus pneumoniae	1.3	5.2	4.9	0.3	0.5	0.4	2.1	2.3	±1.9

All data are given as percentages.
*The sample was tested in duplicate in the second run to assess the intra-assay precision.
BAL, bronchoalveolar lavage.

Clinical Evaluation of the 16S MG Assay and Case Reports

In the subsequent case reports charts, we present the clinical history and laboratory results with the retrospective 16S MG results obtained during this study.

Case 1: Undiagnosed Neonatal Meningoencephalitis

A 1-day–old full-term boy (spontaneous vaginal delivery) presented with sepsis, seizures, apnea, and respiratory failure. The delivery was complicated by a true knot in cord and nuchal cord, and he required oxygen blow for cyanosis. He was empirically treated with gentamicin and ampicillin (meningitis dose), and blood cultures were collected immediately. Lumbar puncture was performed on the following day, which showed 52,500 red blood cells and 70 nucleated cells (90% neutrophils); CSF glucose was 53 mg/dL, and CSF protein was 116 mg/dL. Herpes simplex virus PCR results on the CSF, blood, and CSF cultures were all negative. Extensive genetic workups and metabolite tests were performed to rule out genetic abnormality. The patient was given phenobarbital and levetiracetam for seizure control. Ampicillin and gentamicin were continued for 5 days until the blood cultures were finalized as negative. After 19 days of hospitalization, the seizures and apnea resolved and the patient became progressively more alert and interactive until discharge to home. In the discharge summary, infection was considered an unlikely diagnosis because of the negative microbiological results. On the contrary, we used the 16S MG assay to detect pure *S. intermedius* with 100% abundance in the CSF, suggesting this patient experienced undiagnosed bacterial meningoencephalitis. The negative culture results were most likely due to the empirical antibiotic treatment before the lumbar puncture.

Case 2: Delayed Anaerobic Culture Result in Brain Abscess

A 54-year–old woman presented with 1 week of occipital headache and vision changes due to a right cerebellar abscess. She was admitted to the neurosurgery unit for craniotomy and decompression. Vancomycin, cefepime, and metronidazole were empirically initiated; and the following day, culture results returned *S. intermedius* and *Aggregatibacter aphrophilus*. The treatment was deescalated to ceftriaxone and metronidazole. After 2 days of treatment, the team considered discontinuation of the metronidazole because of a suspected adverse reaction and a lack of anaerobic organisms reported by the laboratory at that point. Two days later, the finalized culture report had an update of the recovery of mixed anaerobic flora, which reassured the necessity of continued metronidazole for the anaerobic coverage. Esophagogastroduodenoscopy identified severe class D esophagitis, which was determined as the source of infection for the brain abscess. Compared with culture, which took 4 days and still could not provide definitive identification of all of the mixed bacteria in the brain abscess, the 16S MG assay provided the results not only quantitatively but also with a much higher resolution: 39% *Fusobacterium nucleatum*, 30% *S. intermedius*, 20% *Capnocytophaga* species, and 7% *A. aphrophilus*.

Case 3: Polymicrobial Infections in Intra-Abdominal Abscess

A 79-year–old woman presented with choledocholithiasis and biliary stricture requiring choledochoduodenostomy. She subsequently developed an intra-abdominal abscess. The bile culture grew mixed Gram-negative rods and a pan-

Table 5 Validation of LOD by Spiking Single Species in Water

		100 CFUs/mL			10 CFUs/mL		
Spiked bacteria	Category	Total reads	Matched reads	Abundance, %*	Total reads	Matched reads	Abundance, %
Escherichia coli	Gram negative	515,436	9066	37.9	585,680	803	0.6
Klebsiella pneumoniae	Gram negative	449,628	14,626	51.4	685,184	ND	ND
Pseudomonas aeruginosa	Gram negative	281,876	22,493	37.8	715,152	3303	8.9
Staphylococcus aureus	Gram positive	391,447	20,116	27.6	234,034	ND	ND
Enterococcus faecalis	Gram positive	598,723	364	1.9	572,751	ND	ND
Clostridium sordellii	Anaerobe	350,556	20,425	48.7	639,644	7437	6.9

*The rest of the reads belonged to background contaminant bacterial DNA.
CFU, colony-forming unit; LOD, limit of detection; ND, not detected.

Table 6 Validation of LOD and Internal Control by Spiking Multiple Species in Body Fluids

			Spiked-in peritoneal fluid (total reads = 1,644,947)		Spiked-in plueral fluid (total reads = 1,515,284)	
Spiked bacteria	Category	Final concentration, CFUs/mL	Matched reads	Abundance, %	Matched reads	Abundance, %
Escherichia coli	Gram negative	100	297,107	60.6	65,432	14.4
Klebsiella pneumoniae	Gram negative	100	31,057	6.4	41,990	9.3
Pseudomonas aeruginosa	Gram negative	100	10,312	2.1	45,914	10.1
Staphylococcus aureus	Gram positive	100	31,419	6.4	55,288	12.2
Enterococcus faecalis	Gram positive	100	2857	0.6	492	0.1
Clostridium perfringens	Anaerobe	100	11,940	2.4	12,631	2.8
Bacteroids spp.	Anaerobe	100	63,883	13	64,083	14.1
Marinobacter spp.	Internal control	500	7708	1.6	91,146	20.1

(*table continues*)

BAL, bronchoalveolar lavage; CFU, colony-forming unit; CSF, cerebrospinal fluid; LOD, limit of detection; ND, not detected.

sensitive *P. aeruginosa*. The Gram-negative rods were so mixed that the laboratory was unable to isolate and identify them. The patient was treated with 10 days of piperacillin-tazobactam, followed by 2 weeks of ciprofloxacin and metronidazole. However, only *P. aeruginosa* was mentioned in the clinical notes for the infection without mention of mixed Gram-negative rods. The 16S MG, however, was able to precisely speciate and quantify the additional pathogens as predominantly *K. pneumonia* (61.7%), which was mixed with 12.8% *Citrobacter freundii*, 9.2% *P. aeruginosa*, 3.4% *E. faecalis*, and 1.2% *Hemophilus parainfluenzae*. In addition, the anaerobe, *Fusobacterium periodonticum* (2.9%), was detected by 16S MG and no anaerobes were detected in culture. In this case, the main etiology of the infection was lost in the electronic medical record, most likely due to minimal speciation in a heavily mixed culture. If full speciation was provided with 16S MG, even without drug susceptibility results, the information could be useful to guide treatment using the hospital antibiogram. Providing the exact species identification with 16S MG instead of a descriptive identification can certainly help better communicate the microbiological results to the clinicians and guide the treatment decision.

Discussion

The use of targeted 16S rRNA gene sequencing, based on the Sanger sequencing technology in culture-negative cases, has been described previously[12]; however, limitations include a lack of sensitivity, inability to detect multiple organisms in complex specimens, and a lack of quantitative analysis of the amount of organism(s) present.[13,14] Herein, we describe an NGS-based quantitative 16S MG assay and analysis platform that allows for both detection and quantification of clinically important pathogenic bacteria. Despite issues with PCR amplification bias and 16S gene copy variation that may obscure the true relative abundance,[15–17] numerous studies have shown that the 16S metagenomics method can achieve reasonable quantification accuracy for complex microbial communities.[18–21] In this study, to minimize the need for ambiguous bases that can lead to amplification bias, the forward primers were designed as a mixture of three different primer variants.[9] Furthermore, amplification bias is more likely to occur when differences are found closer to the 3′-end of the primer. In the forward primers, most ambiguities are in the 5′-end, where less impact is anticipated. Despite these optimization efforts in the primer design, amplification bias may not be eliminated entirely and the relative abundancies of the 16S MG may not always be accurate. Nevertheless, in line with our findings, a recent study by Cummings et al[18] has demonstrated that an NGS-based 16S MG test outperformed standard microbiological culture for characterizing polymicrobial samples with enhanced reproducibility, quantification, and classification accuracy.

In this study, the technical turnaround time (from sample to result) is approximately 2 days. Because of the labor-intensive workflow and high reagent cost, the most cost-effective way of running this test is to batch the samples. An experienced technologist can comfortably run eight clinical samples plus two controls in one batch. The total cost per sample, including reagents, labor, and data analysis, is estimated to be approximately $400 on the basis of a batch of eight samples with two controls. The test can achieve an overall turnaround time of 3 business days if performed twice a week.

The high cost of the 16S MG test relative to culture (20× to 50× more expensive) has limited the broad adoption of

Table 6 (*continued*)

Spiked-in BAL (total reads = 1,172,821)		Spiked-in CSF (total reads = 346,366)		Spiked-in synovial fluid (total reads = 259,923)	
Matched reads	Abundance, %	Matched reads	Abundance, %	Matched reads	Abundance, %
37,381	9.8	ND	ND	ND	ND
ND	ND	ND	ND	ND	ND
30,171	7.9	25,986	27.6	ND	ND
16,036	4.2	17,341	18.4	ND	ND
ND	ND	ND	ND	ND	ND
ND	ND	ND	ND	ND	ND
18,451	4.8	ND	ND	ND	ND
88,796	23.2	26,408	28.1	8999	12.1

this technique, even in highly experienced molecular laboratories. As such, 16S MG is not a replacement for culture but rather a complementary approach, especially in complex or culture-negative cases. As shown by our first case of a neonate with underdiagnosed bacterial meningoencephalitis, the 16S MG test could have provided a more timely diagnosis, resulting in appropriate antimicrobial therapy, avoidance of unnecessary tests and procedures, and a shortened length of hospitalization. In Case 2, the brain abscess was a polymicrobial infection with one anaerobe, *F. nucleatum*, as the most abundant species, which was initially not reported by culture and could have caused the discontinuation of appropriate therapy. Because of their fastidiousness, anaerobes are often missed in many critical infections, such as septic arthritis, osteomyelitis, infective endocarditis, and pneumonia.[22–25] One of the advantages of 16S MG is the ability to detect and identify these anaerobes and simultaneously provide abundance information. In Case 3, a highly polymicrobial infection of the bile duct represented many similar intra-abdominal infections in which more than five bacteria are present and all may be involved in the infection. Traditionally, a microbiology laboratory will only provide a descriptive identification on this kind of polymicrobial culture. The advantage of the 16S MG test includes identification and relative abundance of all the bacteria in polymicrobial infections, which can be used to guide therapy using a local antibiogram. Overall, we have demonstrated that the use of 16S MG for detection and quantification of pathogens in clinical specimens provides a more complete clinical picture in both culture-negative and culture-positive cases.

The US Food and Drug Administration Precision Medicine Initiative has endorsed NGS tests as one of the powerful new approaches for guiding the diagnosis and treatment; researchers involved in this initiative are working on establishing guidelines and standards for NGS-based tests, but these efforts are mainly focused on genetics and oncology rather than infectious diseases (*https://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/PrecisionMedicine-MedicalDevices/default.htm*, last accessed November 2018). Moreover, a significant unmet clinical need in infectious diseases is the diagnostic challenges of patients with signs of infections but negative culture results. NGS-based metagenomics tests have been shown to fill in this vacuum and have been strongly endorsed by both the clinicians and public health practitioners.[26–28] Early adaptors of NGS technology have demonstrated the successes of using the metagenomics tests to diagnose central nervous system infections,[4,6,9,29–31] endocarditis,[32] orthopedic device-related infection,[33,34] and bloodstream infections.[2,31,35] How quickly the NGS-based tests can become available as routine diagnostic tools for infectious diseases will depend on how fast the clinical microbiologists and laboratories can share protocols and establish consensus and standards and how supportive the regulatory bodies, such as the College of American Pathologists and the US Food and Drug Administration, can be to provide oversight and guidelines.

This study has several limitations. First, only a limited number of samples were tested (20 clinical samples and 37 controls or spiked samples). Second, the LOD was estimated by testing only nine bacterial species, which is only a small fraction of the thousands of bacteria that this test is capable of detecting. A lower sensitivity was observed in *Enterococcus* species that are known for their thicker cell walls, which are harder to be ruptured to release DNA.[36,37] Continuous optimization of sample processing techniques, such as prolonged bead beating, is still needed to enhance the sensitivity for those bacteria with thicker cell walls. Third, the linearity and the reportable range of this test were only assessed by samples with two species mixed, which may not be sufficiently mimicking the real clinical samples

in which more than two bacteria species are often present and may change the biochemical dynamics of the 16S rRNA gene amplification. Further study is needed to fully validate the reliability of the quantitative capability of this test when more bacteria species are mixed in the sample. Fourth, the 20% cutoff for filtering out environmental contaminants was empirically determined on the basis of limited data; further study is necessary to ascertain the generalizability of this threshold. Similar to the conventional culture method, the results from the 16S MG test may not conclusively differentiate true infections versus colonization or contamination, and they need to be interpreted carefully and jointly by both clinical microbiologists and infectious diseases specialists. Finally, and most important, the 16S MG test can only detect bacterial pathogens. Other pathogens, including viruses, fungi, and parasites, may also be infectious agents, especially in culture-negative samples.

In summary, we have developed and validated an NGS-based quantitative 16S MG clinical test to aid the diagnosis of bacterial infections in body fluids and tissues. This test has high sensitivity (LOD = 10 to 100 CFUs/mL), wide linearity range (9% to 100% abundance), good precision (95% CI < means ± 10% abundance), and great specificity, with a stringent environmental contaminant filtering algorithm. The turnaround time of this test can be as quick as 48 hours. Compared with culture, the 16S MG test provides many advantages, including detecting pathogens that are culture negative and identifying and quantifying mixed infections, as demonstrated by three clinical cases. This NGS-based quantitative metagenomics test has shown the capability of overcoming current diagnostic challenges in culture-negative and polymicrobial bacterial infections and has the potential to significantly improve patient care and reduce health care cost when used properly.

Acknowledgment

We thank Jesse Kilgore (TriCore Reference Laboratories) for technical assistance in sample preparation.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2019.05.002*.

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