

# Targeted Metagenomic Sequencing-based Approach Applied to 2146 Tissue and Body Fluid Samples in Routine Clinical Practice

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**Background.** The yield of next-generation sequencing (NGS) added to a Sanger sequencing–based 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) assay was evaluated in clinical practice for diagnosis of bacterial infection.

**Methods.** PCR targeting the V1 to V3 regions of the 16S rRNA gene was performed, with amplified DNA submitted to Sanger sequencing and/or NGS (Illumina MiSeq) or reported as negative, depending on the cycle threshold value. A total of 2146 normally sterile tissues or body fluids were tested between August 2020 and March 2021. Clinical sensitivity was assessed in 579 patients from whom clinical data were available.

**Results.** Compared with Sanger sequencing alone (400 positive tests), positivity increased by 87% by adding NGS (347 added positive tests). Clinical sensitivity of the assay that incorporated NGS was 53%, which was higher than culture (42%,  $P < .001$ ), with an impact on clinical decision-making in 14% of infected cases. Clinical sensitivity in the subgroup that received antibiotics at sampling was 41% for culture and 63% for the sequencing assay ( $P < .001$ ).

**Conclusions.** Adding NGS to Sanger sequencing of the PCR-amplified 16S rRNA gene substantially improved test positivity. In the patient population studied, the assay was more sensitive than culture, especially in patients who had received antibiotic therapy.

**Keywords.** clinical metagenomics; 16S ribosomal RNA gene PCR; targeted metagenomics; tissue and body fluids.

Detection and identification of pathogens in infectious diseases are often based on conventional cultures followed by biochemical tests and/or, preferably, matrix-assisted laser desorption ionization time-of-flight mass spectrometry [1]. Cultures have poor sensitivity for fastidious organisms and/or with active or antecedent antimicrobial treatment [2]. This results in culture-negative infections that can be associated with overuse of unnecessarily broad-spectrum antibiotics, exposing patients to adverse effects of antibiotic therapy and selection of resistance or conversely untreated infections and/or delayed diagnoses [3–6]. The last can result in an unfavorable outcome, as shown for endophthalmitis, acute cholecystitis, and sepsis [7–9].

Molecular methods may circumvent these limitations by detecting microbial DNA directly in clinical samples. However, they are typically organism-specific. A broad-spectrum bacterial detection strategy is to target the 16S ribosomal RNA (rRNA) gene using polymerase chain reaction (PCR) followed by sequencing. Amplified product is then sequenced

to identify the source bacterium. When Sanger sequencing is used with this approach, the overall positivity rate is similar to that of conventional cultures [10–12]. Sanger sequencing can generally only read one sequence at a time, restricting analyses to specimens that harbor single bacterial species and limiting sensitivity. In contrast, next-generation sequencing (NGS) can interrogate sequences of large numbers of bacteria at once, theoretically also increasing sensitivity [13]. This approach, an example of targeted metagenomic sequencing, has yielded promising results that suggest that addition of NGS can improve the positivity rate of 16S rRNA gene–based PCR/Sanger sequencing [14–16]. In contrast to shotgun metagenomics (sNGS), targeted metagenomics is not compromised of amplification of human sequences, is subject to facile bioinformatic analysis, and has a shorter turnaround time. However, large-scale experiences with this approach in routine microbiology laboratories have not yet been reported. To reduce labor and the cost of NGS, we developed and implemented in routine practice a targeted metagenomic sequencing–based approach (referred as tNGS) using 16S rRNA PCR followed by Sanger and/or NGS or direct reporting as negative, depending on the PCR cycle threshold (Ct) value. In this retrospective study, the yield of adding NGS to the 16S rRNA-based Sanger sequencing assay in clinical practice and the performance of tNGS compared with culture for infection diagnosis were assessed.

Received 29 December 2021; editorial decision 24 March 2022; published online 1 April 2022.

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Clinical Infectious Diseases® 2022;75(10):1800–8

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## METHODS

### Study Design

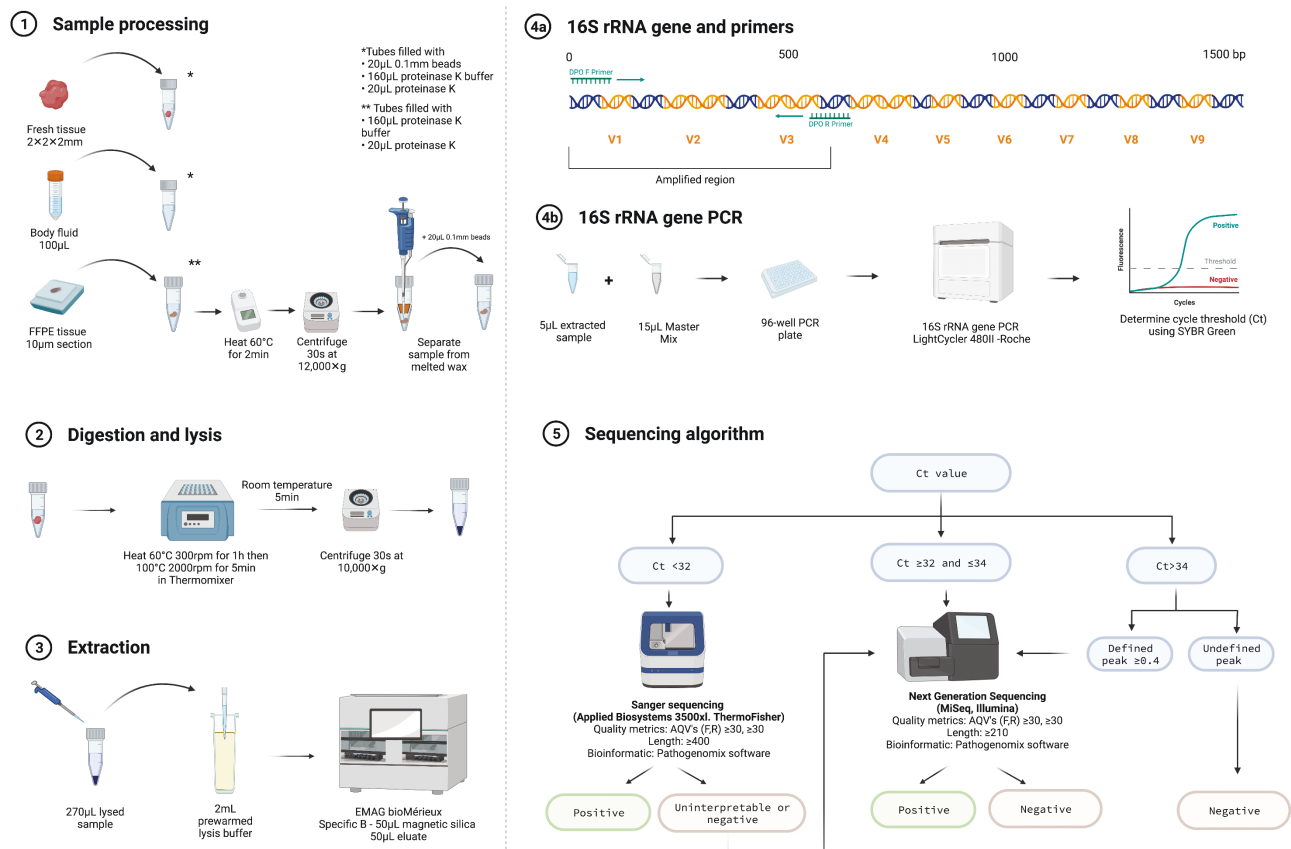
We conducted a retrospective study at a reference laboratory (Mayo Clinic Laboratories) to analyze samples received from 1 August 2020 (assay launch date) to 18 March 2021. Accepted samples were fresh or formalin-fixed paraffin-embedded (FFPE) tissues and body fluids collected from normally sterile sources.

### Targeted Metagenomics Approach

Specimens were processed, lysed, extracted, amplified, and sequenced as shown in Figure 1, along with negative, positive, and inhibition controls (Supplementary Methods). Amplification of the V1 to V3 region of the 16S rRNA gene was performed on a LightCycler 480II (Roche Diagnostics, Risch-Rotkreuz, Switzerland) using dual priming oligonucleotides, as previously described [17]. A Ct value was determined for each

sample. Samples with Ct values <32 cycles were sent to Sanger sequencing. Those with Ct values  $\geq 32$  and  $\leq 34$  or <32 but yielding no identification, poor-quality results, or mixed chromatograms with Sanger sequencing were sent to NGS. Those with Ct values >34 cycles were considered negative and not further analyzed, except if a well-defined melting temperature ( $T_m$ ) peak was observed, in which case they were sent to NGS.

Bidirectional Sanger sequencing was performed on an Applied Biosystems 3500xl instrument (Thermo Fisher Scientific), and NGS was performed on an Illumina Miseq with a 500-cycle (2 × 250 paired-end read) v2 nano kit. Bioinformatic analysis was performed with RipSeq NGS software (Pathogenomix; Supplementary Methods). Doctoral-level clinical microbiologists (R. P. or N. W.) interpreted sequencing results. Organisms considered potential pathogens were reported. Organisms found in the negative control and/or as common assay background and/or across different samples in the same sequencing run were generally not reported.



**Figure 1.** Laboratory workflow of the targeted metagenomics approach (tNGS). (1) A 2-mm<sup>3</sup> piece of tissue or 100 µL of fluid was placed into a lysis tube with 20 µL of 0.1-mm silica/zirconium beads, 160 µL proteinase K buffer (PKB), and 20 µL of proteinase K. For FFPE tissues, a 10-µm section was put into a 1.5-mL centrifuge tube with 160 µL of PKB and 20 µL of proteinase K and heated to 60°C for 2 minutes to melt the wax. After centrifugation, liquid and tissue below the wax were transferred to a lysis tube with 20 µL of 0.1-mm beads. (2) Lysis tubes were incubated/spun, cooled for 5 minutes at room temperature, and then centrifuged. (3) Then, 2 mL of prewarmed (40°C) NUCLESENS easyMAG lysis buffer was added to the disposable NUCLESENS easyMAG cartridge with a maximum of 270 µL of the lysed sample and incubated at room temperature for 10 minutes followed by extraction loading. (4) Next, the 16S rRNA gene polymerase chain reaction targeted the V1–V3 region of the bacterial 16S rRNA gene using dual priming oligonucleotides. (5) Depending on the Ct value, samples were sent to Sanger or next-generation sequencing or reported negative. Abbreviations: AQV, average quality value; Ct, cycle threshold; FFPE, formalin-fixed paraffin-embedded; FR, forward, reverse; PCR, polymerase chain reaction. Created with Biorender.com.

### Clinical Analysis

For clinical sensitivity analysis, samples from Mayo Clinic patients that underwent tNGS and culture were analyzed (Supplementary Figure 1). For both tNGS and cultures, if there was more than 1 sample from the same patient and same source sent to tNGS or culture, only 1 sample was counted, with the result considered positive if at least 1 of the replicates yielded a positive result (except for periprosthetic tissue cultures) and negative if all replicates from the same source were negative. For slow-growing organisms in periprosthetic tissue cultures, 2 positive cultures qualified as positive [18].

Patient and sample characteristics, including clinical and operative notes and laboratory and imaging results, were collected through electronic medical record review. Patients were classified as infected if a culture was positive (and not considered a contaminant) and/or if the treating medical team and the infectious diseases consult team considered the patient to be infected. Culture-negative cases with questionable infection status, observed off antibiotics with improvement were classified as uninfected.

Test impact on clinical decision-making was based on whether tNGS led to antimicrobial de-escalation or escalation and/or continuation or discontinuation of treatment, as recorded in the provider note.

### Cultures

Conventional cultures included at least aerobic and anaerobic cultures and any other cultures (eg, fungal, mycobacterial)

available. Periprosthetic tissue cultures were processed in aerobic and anaerobic blood culture bottles [19].

### Statistics

Qualitative values were compared using the *t* test or Fisher exact test, as appropriate. Quantitative values were tested for normality using the Kolmogorov-Smirnov test and compared using Mann-Whitney or unpaired *t* tests, as appropriate. Clinical sensitivities, specificities, and negative and positive predictive values were calculated using  $2 \times 2$  contingency tables, based on infection status. The 95% confidence intervals were calculated as exact binomial confidence intervals. To compare clinical performance, a McNemar test of paired proportions was performed. *P* values < 0.05 were considered statistically significant.

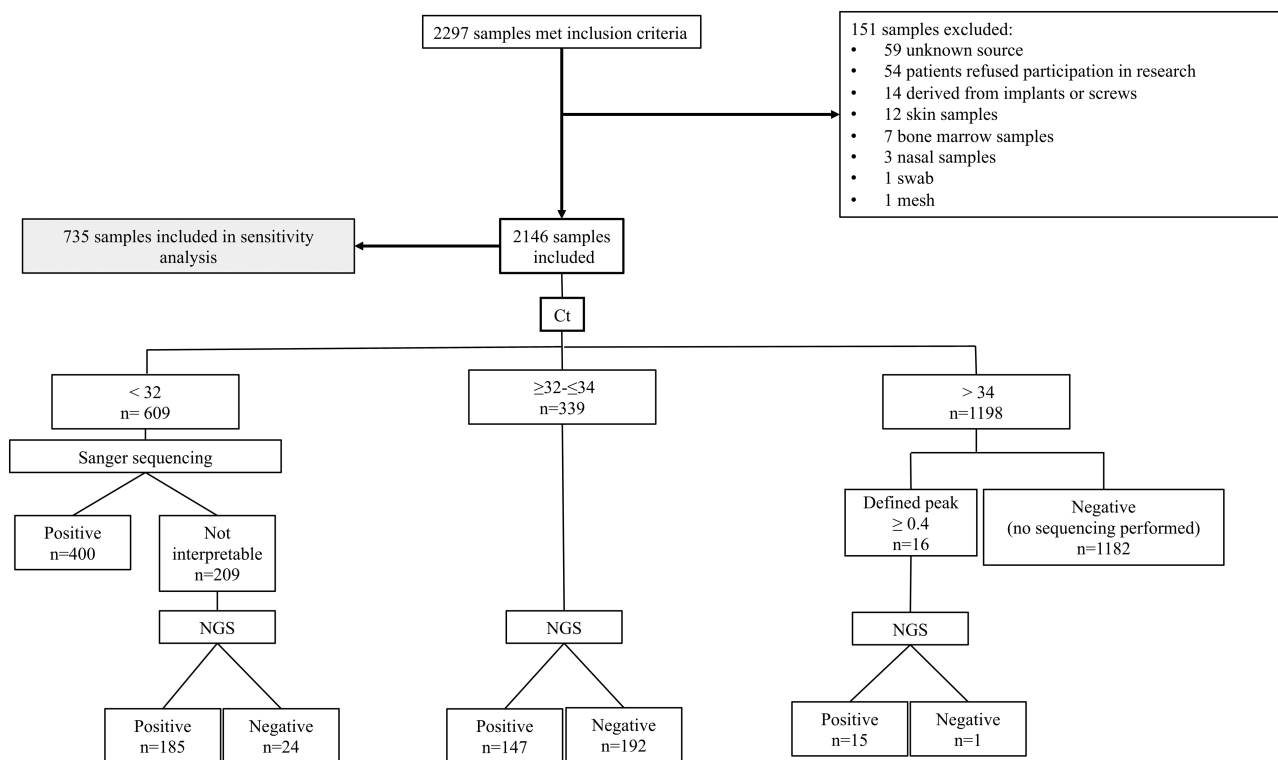
### Ethics

The Mayo Clinic Institutional Review Board approved the study.

## RESULTS

### Samples

A total of 2297 samples met inclusion criteria, of which 151 were excluded. Among the 2146 included samples, 850 (40%) were fluids and 1296 (60%) were tissues (Figure 2). In the group with Ct values <32, 609 (28%) underwent Sanger sequencing,



**Figure 2.** Sample flowchart. Skin and nasal biopsies were excluded as they are not normally sterile sources. Implants, screws, mesh, swabs, bone marrow, and samples of unknown source were excluded. Abbreviations: Ct, cycle threshold; NGS, next-generation sequencing.

resulting in 400 (66%) positive and 209 (34%) uninterpretable results. The latter were sent for NGS, ultimately identifying a potential pathogen in 185 (89%) of the 209. The overall positivity rate in this subset was 96%.

A total of 339 (16%) samples had Ct values between 32 and 34, all of which underwent NGS, with 147 (43%) reported as positive and 192 (57%) negative. A total of 1198 (56%) samples yielded Ct values >34, of which only 16 underwent NGS, with positive and negative results reported in 15 (94%) and 1185 (99%), respectively, of samples in this group.

#### Additional Value of NGS

Overall, the sequencing-based assay was positive in 747 (35%) samples; in 400 (19%) by Sanger sequencing (all monomicrobial) and in 347 (16%) by NGS (140 [40%] polymicrobial and 207 [60%] monomicrobial).

NGS increased positivity by 87% compared with Sanger sequencing alone (98% for tissues and 66% for fluids; [Figure 3A](#)). Increased NGS yield compared with Sanger sequencing was highest for lung tissue (300%), peritoneal fluid (200%), other tissues (231%), bone (174%), and wound biopsies (133%) and was lowest for eye fluids (0%), pericardial fluids (33%), heart valves (37%), cerebrospinal fluid (53%), and synovial fluid (54%). The assay had the highest positivity rate for abscess fluid (77%), abscess tissue (68%), wound biopsies (62%), and heart valves (61%).

#### Microbiology of tNGS-Positive Samples

Nineteen percent of positive samples were polymicrobial. The most common microorganisms detected alone were streptococci (17%), *Staphylococcus aureus* complex (14%), and *Staphylococcus epidermidis* (7%). Difficult-to-grow organisms were found, including mycobacteria (4%), *Bartonella* species (2%), *Nocardia* species (1%), *Ureaplasma* species (0.7%), *Mycoplasma* species (0.5%), and *Tropheryma whipplei* (0.5%; [Figure 3B](#)).

#### Clinical Analysis

##### Sample Characteristics

There were 735 Mayo Clinic patients who met inclusion criteria for clinical analysis. A total of 140 duplicates and 16 samples without culture were excluded, leaving 579 for analysis (337 infected and 242 uninfected; [Supplementary Figure 1](#)). In the infected group, 60 (18%) had fever at sampling; the medians of C-reactive protein in the infected and uninfected groups were 21.5 and 6.9 g/L, respectively ( $P < .001$ ). Overall, 269 (46%) patients had received antibiotics in the 4 weeks before sampling, 214 (64%) and 55 (23%) in the infected and uninfected groups, respectively. In the infected group, 134 (40%) had Ct values <32, with 132 (99%) reported positive; 139 (43%) had Ct values >34 with no Tm peak. In the uninfected group, 211 (87%) had Ct values >34, with 3 (1%) having a Ct value <32 ([Table 1](#)).

#### Impact on Clinical Decision-Making

The tNGS test impacted clinical decision-making in 48 (8%) cases, 46 (14%) and 2 (0.8%) in the infected and uninfected groups, respectively. Treatment de-escalation was the most common impact, occurring in 31 (5%) overall, including 2 (1%) in the uninfected group. Treatment was escalated in 17 (3%), all of whom were infected.

Clinical sensitivity of culture was 42% overall and 40% and 43% in the tissue and fluid groups, respectively. tNGS was more sensitive than culture overall (53%,  $P < .001$ ), in the tissue group (51%,  $P < .001$ ), the fluid group (55%,  $P < .001$ ), and the group that received antimicrobial therapy in the 4 weeks prior to sampling (57%,  $P < .001$ ; [Table 2](#), [Figure 4A](#), [4B](#)). Clinical sensitivity of culture (46%) and tNGS (46%) were comparable ( $P = .9$ ) in the group that had not received antimicrobics in the 4 weeks before sampling; the negative predictive value in this group was 74% for culture and tNGS and 78% with both tests combined. Consideration of both methods together increased clinical sensitivity to 59% overall, with 97% specificity. The increased yield of tNGS over culture was especially marked in patients receiving antibiotics at sampling (sensitivity: 41% for culture, 63% for tNGS, and 70% for the combination thereof; [Figure 4C](#)). Differential sensitivity between the 2 tests generally decreased as the number of antibiotic-free days before sampling increased. The probability of a positive test was higher with tNGS compared with culture over the duration of antimicrobial therapy exposure ( $P = .009$ ; [Figure 4D](#)). In the infected group, 72 (37%) culture-negative samples were positive by tNGS ([Supplementary Figure 2C](#)).

#### Concordance

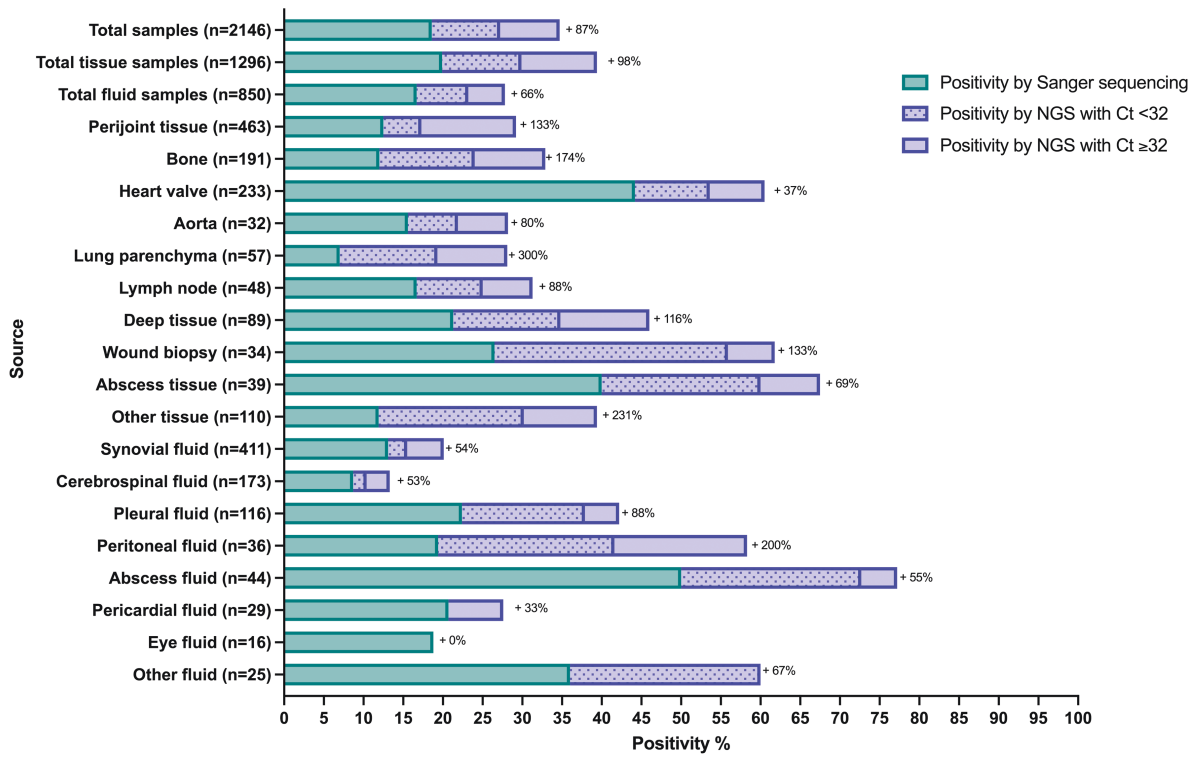
Results (positive or negative) were concordant between culture and tNGS in 81% of cases. In the infected group, discordance between tNGS and cultures was found in 106 samples (31%), with 72 being tNGS-positive/culture-negative and 34 being tNGS-negative/culture-positive ([Supplementary Figure 2](#)). A total of 125 samples in the infected group (37%) were both tNGS- and culture-negative, among which 96 (28%) yielded no microorganism by any testing performed ([Supplementary Figure 2B](#), [2C](#)).

## DISCUSSION

For the past decade, metagenomic testing for infectious diseases has generally been reserved for research and challenging clinical cases, mainly because of cost and technical difficulty but also because of the uncertainty as to clinical value. This real-life study provides insight as to how tNGS implemented in a high-throughput clinical molecular microbiology laboratory yields benefits in terms of pathogen detection in infection diagnosis and impacts patient care. Addition of NGS increased 16S rRNA gene PCR positivity by 87% compared with Sanger sequencing alone. tNGS positivity was 35% higher than the 13% (78 of 607) and 11% (35 of 312) positivity of Sanger sequencing alone that

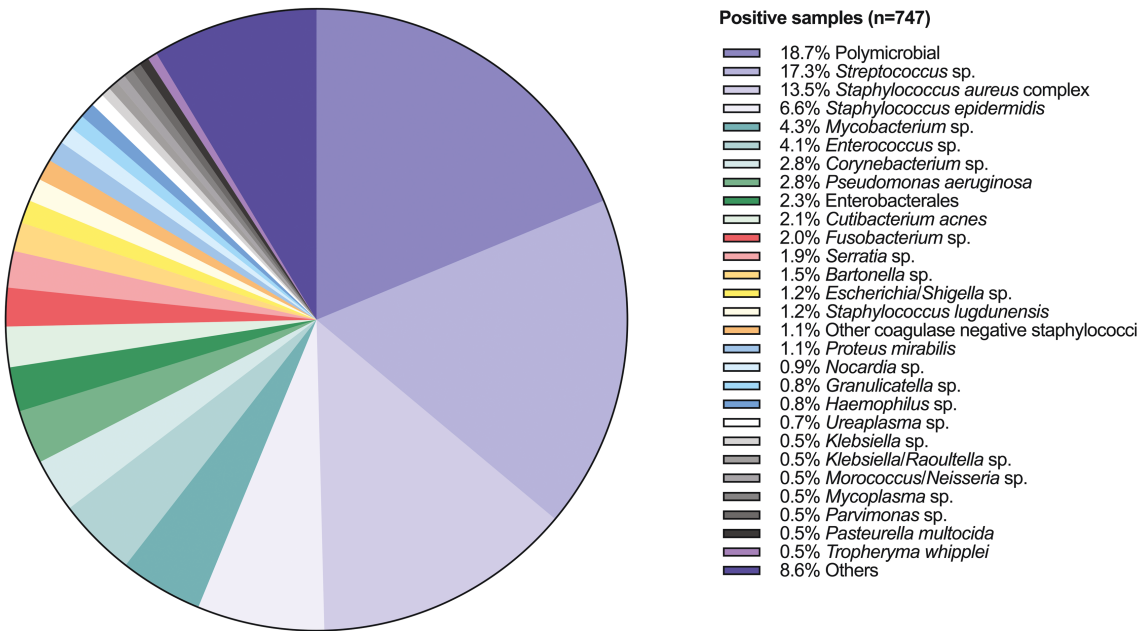


### Yield of next-generation sequencing (NGS)



A.

### Identification by targeted metagenomics



B.

**Figure 3. Additional value of targeted metagenomics (tNGS).** A, The x-axis is the positivity rate of the test (calculated as the number positive by the tNGS assay divided by the total number of samples in each group on the y-axis). Incremental yield of the tNGS assay offered by NGS was calculated as the number of positive results by NGS divided by the number of samples positive by Sanger sequencing and displayed as +XX% next to each bar group. The “other tissue” group includes biopsies of serous membranes, muscle, organs (brain, liver, spleen, kidney, ovary, breast), digestive tract, masses or tumors not identified as abscesses, thrombi, and deep tissues from pacemaker pockets. The “other fluid” group includes mediastinal fluid, abdominal fluid (including bile), and any fluid not identified as being from an abscess. B, Descriptive microbiology of the microorganisms sequenced by tNGS in the 747 positive samples. Of the 607 monomicrobial samples, 529 (87%) were identified to the species level and 78 (13%) to the genus level only. For formatting reasons, some bacteria are grouped by genus in the figure, even if identified to the species level. Abbreviations: Ct, cycle threshold; NGS, next-generation sequencing.

**Table 1. Patient Characteristics at the Time of Sampling**

Characteristic	Total samples n = 579	Infection n = 337	No infection n = 242	PValue
	n (%)	n (%)	n (%)	
Tissue samples	281 (49)	214 (64)	67 (28)	
Fluid samples	298 (51)	123 (36)	175 (72)	
Abnormal temperature (>38.5°C or <36°C)	72 (12)	60 (18)	12 (5)	<0.001
Peripheral blood leukocytes (G/L), n = 532	7.9 (6.2–10.3)	8.5 (6.6–11.4)	7.2 (5.9–9.3)	<0.001
C-reactive protein (g/L), n = 473	21.5 (4.5–60.2)	38.1 (10.2–85.1)	6.9 (0–23.4)	<0.001
Erythrocyte sedimentation rate (mm/h), n = 426	27 (9–55)	36 (16–73)	21 (5–40)	<0.001
Gram stain	n = 418	n = 268	n = 150	-
Positive Gram stain	24 (6)	22 (8)	2 (0.8)	0.003
Conventional cultures				
Positive cultures	141 (24)	140 (42)	1 (0.4)	-
Negative cultures	438 (76)	197 (58)	241 (99.6)	-
tNGS				
Positive	183 (32)	178 (53)	5 (2)	-
Negative	396 (68)	159 (47)	237 (98)	-
Samples with Ct <32	137 (24)	134 (40)	3 (1)	-
Positive with Sanger sequencing	105 (77)	104 (77)	1 (33)	-
Positive with NGS/number sent to NGS	29/32 (91)	28/30 (93)	1/2 (50)	-
Samples with Ct ≥32 to ≤34	86 (15)	58 (17)	28 (12)	-
Positive with NGS/number sent to NGS	45/86 (52)	42/58 (72)	3/28 (11)	-
Samples with Ct >34	356 (61)	144 (43)	211 (87)	-
Positive with NGS/number sent to NGS	4/5 (80)	4/5 (80)	0/0	-
Antibiotics administered in the 4 weeks before test	269 (46)	214 (64)	55 (23)	-
Impact on clinical decision-making	48 (8)	46 (14)	2 (0.8)	-
Escalation of treatment	17 (3)	17 (5)	0 (0)	-
De-escalation of treatment	31 (5)	29 (9)	2 (0.8)	-
Concordant positivity/negativity between tNGS and cultures	469 (81)	231 (69)	238 (98)	-
tNGS positive and culture positive	107 (19)	106 (31)	1 (0.4)	-
tNGS positive and culture negative	76 (13)	72 (21)	4 (2)	-
tNGS negative and culture positive	34 (6)	34 (10)	0 (0)	-
tNGS negative and culture negative	362 (56)	125 (37)	237 (98)	-

Abbreviations: Ct, cycle threshold; NGS, next-generation sequencing; tNGS, targeted metagenomic sequencing–based approach.

Characteristics at the time of sampling were recorded. Medians and 25–75 interquartile ranges are displayed for quantitative values. Numbers and percentages are displayed for qualitative values.

were reported by Aggarwal et al [10] and Yoo et al [20], respectively. In addition, tNGS identified multiple difficult-to-grow organisms, confirming its use for infection types associated with delayed diagnoses [21].

Some limitations of Sanger sequencing were circumvented with NGS, in particular, detection of polymicrobial samples (which represented 19% of positive samples in this study). For sample types with a high likelihood of polymicrobial results, such as lung tissue, peritoneal fluid, wound samples, and pleural fluid, Sanger sequencing sometimes failed in more than half of cases, even with low Ct values, requiring NGS such that direct NGS regardless of the Ct value deserves further study. Advantages of using NGS for such specimen types have been highlighted by others [16, 22–24].

This study is the first, to date, to describe the clinical performance of a tNGS approach using a large number of samples, with results showing that tNGS has higher clinical sensitivity

than cultures (albeit in a study population likely biased to culture negativity), with high specificity (98%) and positive and negative predictive values of 97% and 60%, respectively.

An alternative to tNGS is sNGS. One study evaluated sNGS in a clinical setting using 109 samples of several types and reported a sensitivity and specificity of 67% and 69%, respectively [25]. Others studied sNGS on plasma and lower respiratory tract specimens and reported high sensitivities (>90%), albeit with low specificities of 63% [26] and 42% [27], respectively. False-positive results have possible consequences in terms of overprescription of antimicrobial agents and missed/delayed diagnosis of “real” diagnoses. Gu et al evaluated sNGS to detect bacteria in body fluids and reported a sensitivity of 79% and specificity of 91%, but only on samples with a confirmed diagnosis by culture or PCR [28]. In our cohort, we calculated clinical sensitivity, with 197 culture negative samples, including 96 that yielded no pathogen identification by any other mean.

**Table 2. Clinical Analysis of the Targeted Metagenomics Sequencing-based Approach and Cultures**

	Sensitivity, % (95% CI interval)	Specificity, % (95% CI)	Negative Predictive Value, % (95% CI)	Positive Predictive Value, % (95% CI)
All samples, n = 579				
Cultures	42 (37–47)	100 (98–100)	55 (50–60)	99 (96–100)
Targeted metagenomics	53 <sup>a</sup> (47–58)	98 (95–99)	60 (55–65)	97 (94–99)
Cultures and/or targeted metagenomics	63 <sup>a</sup> (58–68)	98 (95–99)	65 (60–70)	98 (95–99)
Tissue samples, n = 281				
Cultures	40 (33–46)	99 (92–100)	34 (28–41)	99 (94–100)
Targeted metagenomics	51 <sup>a</sup> (45–58)	97 (90–99)	38 (31–46)	98 (94–100)
Cultures and/or targeted metagenomics	64 <sup>a</sup> (57–70)	97 (90–99)	46 (38–54)	99 (95–100)
Fluid samples, n = 298				
Cultures	43 (35–52)	100 (98–100)	71 (65–77)	100 (93–100)
Targeted metagenomics	55 <sup>a</sup> (46–64)	98 (95–100)	76 (70–81)	96 (88–99)
Cultures and/or targeted metagenomics	61 <sup>a</sup> (52–69)	98 (95–100)	78 (72–83)	96 (89–99)
Antibiotics received administered in the 4 weeks before sampling, n = 269				
Cultures	39 (33–46)	98 (90–100)	29 (23–36)	99 (94–100)
Targeted metagenomics	57 <sup>a</sup> (50–63)	95 (85–99)	36 (29–44)	98 (93–99)
Cultures and/or targeted metagenomics	65 <sup>a</sup> (59–71)	95 (85–99)	41 (33–50)	98 (94–99)
No antibiotics received administered in the 4 weeks before sampling, n = 310				
Cultures	46 (37–54)	100 (98–100)	74 (68–79)	100 (94–100)
Targeted metagenomics	46 (38–55)	99 (96–100)	74 (68–79)	97 (88–99)
Cultures and/or targeted metagenomics	59 <sup>a</sup> (50–67)	99 (96–100)	78 (73–83)	97 (91–100)

Abbreviation: CI, confidence interval.

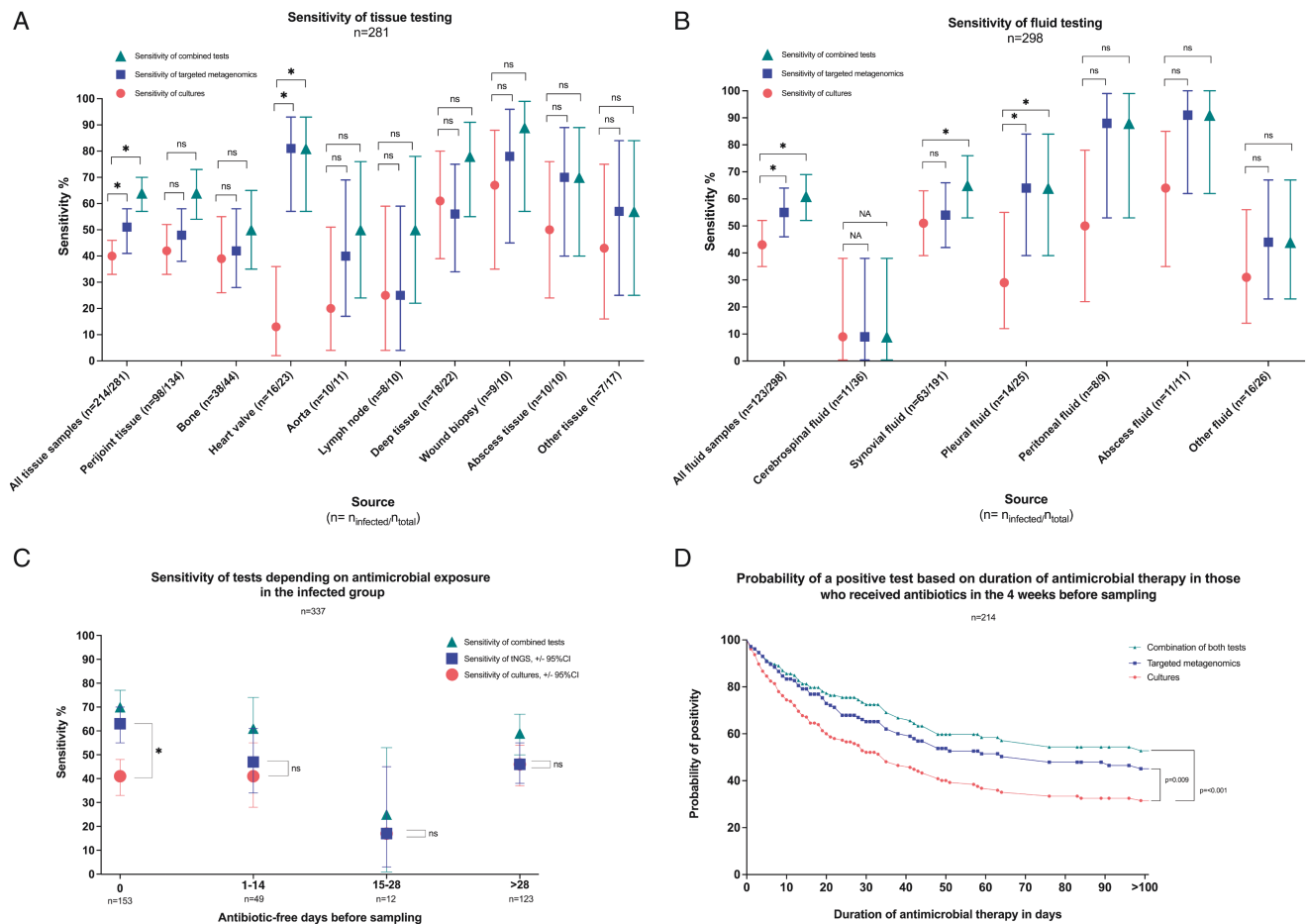
<sup>a</sup> *P* < .001 compared to cultures.

sNGS is, however, theoretically better suited than 16S rRNA gene-based tNGS for infections where viruses and/or fungi are dominant, such as meningitis, meningoencephalitis, and eye infections [29, 30]. Addition of a conserved fungal gene into a tNGS approach may be helpful.

tNGS had an impact on clinical decision-making for 8% of samples overall, 14% in the infected group and 0.8% in the uninfected group. Clinicians might have been cautious about changing management since this was a new assay. These numbers are comparable to those from a French study on 16S rRNA PCR/Sanger sequencing where an impact on clinical decisions was found in 62 of 806 (8%) overall and 62 of 109 (32%) positive samples [31]. The effect of antimicrobial therapy on tNGS was also evaluated. tNGS was more sensitive than culture among patients who received antimicrobial treatment at sampling. This difference decreased over time as the number of antibiotic-free days increased. Clinical sensitivity of tNGS was higher than culture among patients with prolonged antibiotic exposure.

There are limitations of this study. In the infected group, 43% of samples were not sequenced because they had a Ct >34. Use of Ct values to drive the sequencing trajectory was intentional to avoid sequencing samples with a low chance of positivity, reducing labor, cost, and the potential for false-positive results. In previous research, a threshold Ct of 34 was proposed to differentiate positive and negative samples, with specific application to arthroplasty-derived sonicate fluids [15]. This

cutoff might not apply equally to all sample types and therefore should be evaluated by sample source and infection type. That there were diverse sample types in this study is also a limitation. The definition of infection was based on assessment of the clinical team. Despite being imperfect, this represents real life and has been previously used to assess clinical sensitivity of metagenomic tests in infectious diseases [32]. Providers were not blinded to results of tNGS. Consequently, for patients with negative cultures and doubtful presence of infection, tNGS negativity may have made providers more likely to observe patients off antibiotics. Clinical sensitivity was calculated because the aim was to correlate the presence of infection with results of the assay; hence, the low sensitivity. Interpretation of results requires a trained expert who can adjudicate results and understand assay background. *Cutibacterium acnes* and *S. epidermidis*, which may be contaminants or pathogens, were not typically reported as pathogens if found in negative controls. While a cost analysis was not performed, the cost of tNGS is several-fold less than that of sNGS [33, 34]. Theoretical turnaround time is dependent on Ct value, with a result in less than 24 hours for negative samples or those sent to Sanger sequencing, and 36–48 hours (ideally) for samples sent to NGS. However, maintaining a 48-hour turnaround time for NGS in clinical practice is challenging, with time to delivery of results being as long as 10 days in some cases. To improve time to results, trained technologists would need to



**Figure 4.** Clinical sensitivity by sample type and antimicrobial exposure. *A*, Clinical sensitivity (determined as the percentage of positive identifications in the infected group) of cultures, tNGS, and the combination of the 2 for tissue samples by source. *B*, Clinical sensitivity (percentage of positive identifications in the infected group) of cultures, tNGS, and the combination of the 2 for fluid samples by source. *C*, Sensitivity (percentage of positive identifications in the infected group) by numbers of days without antimicrobial therapy prior to sampling. Sensitivities of cultures and tNGS were compared using the McNemar test of paired proportions. \**P* value < .05. *D*, Kaplan-Meier curve showing probability of positivity for cultures, tNGS, and both based on duration of antimicrobial therapy before sampling in the infected group (n = 214). Patients who had received more than 100 days of antimicrobial therapy prior to sampling were included in the analysis but were censored at 100 days on the graphic representation. The probability of culture positivity was compared with tNGS and the combination of the 2 using a log-rank test (Mantel-Cox). Abbreviations: CI, confidence interval; ns = nonsignificant; tNGS, targeted metagenomics.

be available 24/7 to perform NGS. Nevertheless, turnaround time of cultures for certain organisms such as mycobacteria may be longer than 10 days tNGS can still be clinically relevant. Performance of the test on FFPE compared with fresh tissue, which is a controversial subject, was not assessed [35, 36]. Finally, because a 16S rRNA PCR approach was used, prediction of antibiotic resistance was not possible. The tNGS test that was studied was only used for normally sterile human tissues and body fluids. Sampling of plasma or whole blood, particularly for acutely ill patients or those for whom the site of infection is not known [37], or for diagnosis of tick-borne pathogens, immunocompromised patients, or septic patients [38–41], or sampling of other specimens, such as respiratory secretions or skin, requires further study.

In conclusion, results of this study show the benefit of adding NGS to a Sanger sequencing–based 16S rRNA PCR assay and overall value of tNGS in a clinical setting. This approach is

not meant to replace culture but to serve as a complementary diagnostic tool for fastidious bacteria, patients who receive antibiotics before sampling, and/or to tease apart undefined etiologies of potential infections.

#### Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyrighted and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

#### Notes

**Disclaimer.** The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health (NIH).

**Financial support.** Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the NIH under R01AR05664.

**Potential conflicts of interest.** R.P. reports grants from ContraFect, TenNor Therapeutics Limited, and BioFire; is a consultant to Curetis, Specific



Technologies, Next Gen Diagnostics, PathoQuest, Selux Diagnostics, 1928 Diagnostics, PhAST, Torus Biosystems, Day Zero Diagnostics, Mammoth Biosciences, and Qvella; monies are paid to Mayo Clinic. Mayo Clinic and R.P. have relationships with Adaptive Phage Therapeutics and Pathogenomix. R.P. is a consultant to Netflix and CARB-X; has a patent on *Bordetella pertussis/parapertussis* polymerase chain reaction issued, a patent on a device/method for sonication with royalties paid by Samsung to Mayo Clinic, and a patent on an antibiofilm substance issued; receives honoraria from the National Board of Medical Examiners, Up-to-Date, and the Infectious Diseases Board Review Course; reports being Chair, ASM Governance Committee and Member, Finance Committee (ASM); and reports an editor's stipend from the Infectious Diseases Society of America. N.W. reports royalties or licenses from Roche Diagnostics; and a leadership or fiduciary role for Clinical and Laboratory Standards Institute working groups and document development committees. All authors are employees of Mayo Clinic. M.W. reports having a relationship with Pathogenomix through Mayo Clinic. All remaining authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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