NGS in the clinical microbiology settings

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We hypothesized that targeted NGS sequencing might have an advantage over Sanger sequencing, especially in polymicrobial infections. The study included 55 specimens from 51 patients. We compared targeted NGS to Sanger sequencing in clinical samples submitted for Sanger sequencing. The overall concordance rate was 58% (32/55) for NGS vs. Sanger. NGS identified 9 polymicrobial and 2 monomicrobial infections among 19 Sanger-negative samples and 8 polymicrobial infections in 11 samples where a 16S gene was identified by gel electrophoresis, but could not be mapped to an identified pathogen by Sanger. We estimated that NGS could have contributed to patient management in 6/18 evaluated patients and thus has an advantage over Sanger sequencing in certain polymicrobial infections.

KEYWORDS
NGS, 16s, clinical microbiology, next generation sequencing, polymicrobial infections, polymicrobial

Introduction

Most clinical infections are not microbiologically confirmed. The problem is especially pertinent in deep seated invasive infections, where microbiological diagnosis is critical and the specimen is precious, such as osteomyelitis, deep organ abscesses, brain empyema or others. For these cases, there has been a growing interest and implementation of broad-range polymerase chain reaction (PCR) based Sanger sequencing of the 16S ribosomal RNA (rRNA) bacterial gene (panbacterial PCR), directly from clinical specimens (Rampini et al., 2011). Sanger sequencing significantly improved the diagnostic yield in clinical culture isolates as well as mono-microbial infections (Shachor-Meyouhas et al., 2013; Khoury et al., 2019). However, when more than one species are present in the specimen, Sanger sequencing template reads are superimposed and are generally uninterpretable (Salipante et al., 2013). Results from
such specimens are reported as negative or as a mixture of bacteria, without further identification.

As in other fields of medicine, next generation sequencing (NGS) technologies have expanded diagnostic capabilities in clinical microbiology laboratories. Recent studies have highlighted the ability of 16S rRNA NGS to accurately reach speciation and quantify bacterial abundances in complex polymicrobial infections (Salipante et al., 2013; Abayasekara et al., 2017; Culbreath et al., 2019).

We hypothesized that for difficult-to-diagnose infections, especially when polymicrobial, targeted NGS of the 16S rRNA gene has better diagnostic performance than panbacterial Sanger sequencing.

Materials and methods

We used residual nucleic acids from clinical specimens that were submitted to our reference molecular laboratory at RHCC for panbacterial, panfungal or mycobacterial PCR (Sanger sequencing). Samples were collected between 2020-2021 at Rambam Health Care Campus (RHCC) or other hospitals. DNA stored at -20°C was retrieved and tested by NGS retrospectively. Clinical information was available only for samples sent from within RHCC.

Broad-range 16S rRNA gene Sanger sequencing was performed using in-house protocols at the molecular bacteriology laboratory (Shachor-Meyouhas et al., 2013). NGS library preparation and analyses were performed blinded to clinical information, culture or Sanger sequencing results. DNA extraction of bacterial DNA from each specimen was carried out using the QIAAmp™ DNA Mini kit (QiagenGroup) according to manufacturer’s instructions. Each batch of specimens were extracted with negative controls (extraction control). PCR amplification of the hypervariable region V4 of the 16s rRNA gene was conducted using PCRBIO HS Taq Mix Red according to the Earth Microbiome Project primer pairs (Apprill et al., 2015; Parada et al., 2016), and in selected cases, the addition of V1-2 segments (F27-R338) of the same gene to better identify certain bacteria and improve speciation (such as for Staphylococcus and Enterobacterales) (Walker et al., 2015). PCR products were run on a 1.5% agarose gel. Final library products were purified using Qiaquick PCR Purification Kit (Qiagen Groups) according to manufacturer’s instructions and quantified using Qubit™ dsDNA HS and BR Assay Kits (Invitrogen).

We sequenced the amplified V1-2 or V4 regions of 16s rRNA gene with Ion SS™ System (Thermo Fisher Scientific). Data were analyzed using the Ion Reporter bioinformatics Software pipeline (Thermo Fisher Scientific), using a threshold of 1000 mapped reads for designating significant pathogens. BAM files uploaded to the Ion Reporter were mapped to the Silva 138 SSU database. Typical contaminants found in negative controls, such as Acinetobacter Iwoffi, Acinetobacter schendi, or Xanthomonadaceae which are water tolerant bacteria, were subtracted from overall reads obtained on the clinical samples.

Concordance between Sanger sequencing and NGS results was evaluated. For the RHCC samples, we estimated the potential clinical added value of NGS, had it been available in real time. Two physicians (infectious diseases and clinical microbiologist) evaluated each case and assigned the potential contribution of NGS to patient management independently (diagnosis and treatment). Disagreements were resolved by consensus.

The study was approved by the local ethics committee with a waiver of informed consent. NGS results were not conveyed to clinicians.

Results

The study included 55 specimens from 51 patients; 22 specimens from RHCC and all others from other hospitals. Of the 55 specimens evaluated, 25 specimens were Sanger positive with one organism reported. Of those, 24 were concordant by NGS (24/25) that identified the exact Sanger pathogen alone (N=12) or with additional pathogen/s (N=12). The only discordant result was a Propionibacterium spp identified by Sanger, that was missed by NGS. Sanger was negative in 19 samples, of which 8 were negative by NGS, 9 polymicrobial and 2 monomicrobial by NGS. In 11 samples, broad-range 16S gene was identified by gel electrophoresis, but could not be mapped to an identified pathogen within available databases when sequenced by Sanger technology (possible polymicrobial infection); among these, 3/11 were negative on NGS and all others were positive with polymicrobial identification (Figure 1 and Table 1).

Among the 22 RHCC specimens for which clinical information was available (18 patients), eight could have benefited from NGS for diagnosis (Figure 2 and Table 2): 4 polymicrobial (NGS-26, 30, 32, 34), 3 monomicrobial findings (NGS-23, 27, 54) and 1 genus identification of E.coli/Salmonella spp. in specimen NGS-44. We estimated that 6/18 patients would have benefited from antibiotic adjustment following NGS results and they all belong to the discordant group (Figure 2 and Table 2), while the other 12 patients would have been treated properly without any change in empiric treatment.

In this study we describe the comparison between broad range Sanger panbacterial sequencing and targeted deep sequencing (NGS) on clinical samples submitted for panbacterial PCR. Overall, the concordance rate was 58% (32/55) for NGS vs. Sanger. Concordance was more frequent in Sanger-positive samples 24/25 (96%) than for Sanger-negative 8/19 (42%). Among five discordant Sanger-negative results with clinical information (Table 2), the positive NGS result would
**FIGURE 1**
Concordance between Sanger sequencing and NGS in a scheme. Sanger negative are those samples that did not give any signal on PCR gels. “Sanger unidentified” are those samples that presented a band in agarose gels, but on Sanger sequencing it was not possible to define a unique organism against public databases.

**TABLE 1**  Comparison between Sanger and NGS results.

| Sample ID | Material | Sanger results | NGS results | Total number of mapped reads |
|-----------|----------|----------------|-------------|-----------------------------|
| NGS-1     | Tissue/ skin/soft tissue | *Pseudomonas aeruginosa* | *Pseudomonas* spp 28% (*Pseudomonas aeruginosa* 2%) | 7946 |
| NGS-14    | Pus | *Pseudomonas aeruginosa* | *Pseudomonas* 74% (*P.aeruginosa* 39%) | 18412 |
| NGS-15    | Pus | *Fusobacterium necrophorum* | *Fusobacterium necrophorum* 34% | 16616 |
| NGS-2     | Wound | *Mycobacterium marinum* | *Mycobacterium* spp 20% | 4208 |
| NGS-20    | Tissue | *Staphylococcus aureus* | *Staphylococcus* spp 71% (S. epidermidis 11%, S. aureus 3%) | 9765 |
| NGS-3     | Tissue | *Streptococcus pyogenes* | *Streptococcus* pyogenes 27% | 4929 |
| NGS-33    | Blood | *Sneathia sanguinegens* | *Sneathia sanguinegens* 60% | 201717 |
| NGS-39    | Tissue | *Staphylococcus aureus* | *Staphylococcus* spp 72% (S. epidermidis 9%, S. aureus 1.5%) | 2609 |
| NGS-40    | lymph node | *Streptococcus pyogenes* | *Streptococcus* pyogenes 35% | 1440 |
| NGS-46    | Pleural Fluid | *Enterococcus spp* | *Enterococcus* spp 29% (*E. moraviensis* 1%) | 1569 |
| NGS-7     | Tissue | *Streptococcus dysgalactiae spp equisimilis* | *Streptococcus dysgalactiae* 30% | 13866 |

(Continued)
| Sample ID | Material | Sanger results | NGS results | Total number of mapped reads |
|-----------|----------|----------------|-------------|----------------------------|
| NGS-9     | Tissue   | *Staphylococcus aureus* | *Staphylococcus aureus* | 2107 |
| NGS-32    | Swab     | *Streptococcus sanguis* | Polymicrobial (Rothia mucilaginosa 10%, Velinella dispar 12%, Prevotella 11% (P. histicola 5%, P. salivae 3%, others <1%) Streptococcus 35%, (S. australis 6%, S. infantis 5%), Actinomadura parahemolytica 1%) | 2038 |
| NGS-36    | BAL      | *Streptococcus mitis* | Polymicrobial (Rothia mucilaginosa 28%, Streptococcus 46% (S. australis 6%, S. infantis 8%), Velinella <1%) | 4870 |
| NGS-53    | Swab     | *Haemophilus influenzae* | *Haemophilus influenzae* | 1737 |
| NGS-11    | Fluid    | *Porphyromonas spp* | Polymicrobial (Porphyromonas endodontalis 25%, Bacteroides fragilis 5%) | 10198 |
| NGS-12    | Pus      | *Enterobacteriaceae* | Polymicrobial (Enterobacter 3%, Klebsiella 2% , Enterococcus 3%) | 20144 |
| NGS-25    | Bronchial wash | *Pseudomonas spp* | Polymicrobial (Prevotella 15% (P. histicola11%, P.melaninogenica3%, P. veronael 1%), Velinella dispar 7%, Pseudomonas spp 16%, Streptococcus spp 23 (S.infantis 1%anginosus, australis, thermophilus <1%)) | 54561 |
| NGS-44    | Abscess  | *Enterobacteriaceae* | Salmonella 3%, Escherichia 2% | 699 |
| NGS-49    | Tissue   | *Proteus spp* | Polymicrobial (Peptophilus 23%, Finegoldsia magna 1%) | 889 |
| NGS-50    | Tissue   | *Finegoldsia spp* | Polymicrobial (Peptophilus 9%, Finegoldsia magna 23%, Prevotella timonensis 10%, Anaerococcus murochii 5%) | 1614 |
| NGS-51    | Swab     | *Capnocytophaga spp* | Polymicrobial (Granulicatella adiacens 1%, Capnocytophaga leadbetteri 8% , Fusobacterium periodonticum<1%, Rothia mucilaginosa 4%, Neisseria cinerea15%, Haemophilus parainfluenzae 8%, Prevotella nanenciens 3%, Streptococcus australis , Streptococcus infantis , Veillonella) | 7495 |
| NGS-6     | Pus      | *Morganella spp* | Polymicrobial (Pseudomonas spp 39% (Pseudomonas aeruginosa 16%)) | 4772 |
| NGS-8     | Pus      | *Prevotella spp* | Polymicrobial (Prevotella melaninogena 19%, Finegoldsia magna 10%,Vellionella spp 10%, Gemella spp 4%) | 18344 |

**Concordant, negative (N=8)**

| Sample ID | Material         | Sanger results                          | NGS results                                                                 | Total number of mapped reads |
|-----------|------------------|-----------------------------------------|------------------------------------------------------------------------------|----------------------------|
| NGS-18    | Tissue           | Negative                                | Negative                                                                      | 551 |
| NGS-19    | Synovial Fluid   | Negative                                | Negative                                                                      | 46968 |
| NGS-21    | Synovial Fluid   | Negative                                | Negative                                                                      | 29735 |
| NGS-22    | Synovial Fluid   | Negative                                | Negative                                                                      | 0 |
| NGS-24    | Tissue biopsy, abscess | Negative                                  | Negative                                                                      | 428 |
| NGS-29    | CSF, surgical site | Negative                                | Negative                                                                      | 494 |
| NGS-35    | CSF              | Negative                                | Negative                                                                      | 900 |
| NGS-43    | Fluid            | Negative                                | Negative                                                                      | 1211 |

**Discordant, Sanger-negative, N=11**

| Sample ID | Material | Sanger results | NGS results                                                                 | Total number of mapped reads |
|-----------|----------|----------------|------------------------------------------------------------------------------|----------------------------|
| NGS-13    | Tissue   | Negative       | Polymicrobial (Streptococcus 20% S. infantis 1%, S. australis 1%, Granulicatella elegans 2%, Gemella spp 4%, Haemophilus parainfluenzae 3%, Neisseria 1%, Rothia mucilaginosa 2%, Prevotella melaninogena) | 7668 |

(Continued)
| Sample ID | Material            | Sanger results | NGS results                                                                 | Total number of mapped reads |
|-----------|---------------------|----------------|------------------------------------------------------------------------------|-----------------------------|
|           |                     |                | 6%                                                                           |                             |
|           |                     |                | V1-2: Helicobacter pylori 19%                                                 |                             |
| NGS-16    | Synovial fluid      | Negative       | Polymicrobial (Porphyromonas uenonis 3%, Prevotella oris 6%, Parvimonas micro 5%, Enterococcus hominis 3%, Fusobacterium 5%) |                             |
| NGS-17    | Pleural fluid       | Negative       | Polymicrobial (Prevotella oris 1%, Parvimonas micro 1%, Fusobacterium 3%)    |                             |
| NGS-19    | Tissue              | Negative       | Polymicrobial (Porphyromonas uenonis 1%, Parvimonas micro 2%, Fusobacterium 2%, Prevotella 2% oris and oralis) |                             |
| NGS-23    | Abscess             | Negative       | Staphylococcus 20% (S.aureus <1%, S. epidermidis 4%)                         |                             |
| NGS-26    | CSF                 | Negative       | Polymicrobial (Staphylococcus 21% (S. epidermidis 2%), Micrococcus 2%, Paenibacillus 5%, Legionella pneumophila 1.6%) |                             |
| NGS-27    | Tissue-brain        | Negative       | Staphylococcus 22% (S. epidermidis 2%)                                       |                             |
| NGS-28    | Tissue              | Negative       | Polymicrobial (Corynebacterium spp 10%, Dermatobacter hominis 2%, Anaerococcus mordaci 2%, Peptostreptococcus 9%, Ethromadum ramosum <1%) |                             |
| NGS-30    | Tissue biopsy       | Negative       | Polymicrobial (Corynebacterium kroppenstedtii <1%, Enterobacteuriaceae 5% (Enterobacter cloacae <1%)) |                             |
| NGS-4     | Wound swab          | Negative       | Polymicrobial (Prevotella oris 1%, Prevotella oris 3%, Parvimonas micro 3%, Fusobacterium 9% (F. nucleatum <1%), Pseudomonas 2% (P.auruginosa <1%), P.ribosolio <1%)) |                             |
| NGS-55    | BAL                 | Negative       | Streptococcus 40% (S. infantis 5%)                                           |                             |
|           |                     |                | V1-2: (very low counts 191): Haemophilus parainfluenza                        |                             |

**Discordant, Sanger-positive, N=1**

| NGS-42   | Surgical wound/abscess | Propionibacterium spp | Negative | 504 |

**Sanger positive but unidentified (N=11)**

| NGS-31   | Pus                  | Positive, unidentifed | Negative | 817 |
| NGS-38   | BAL                  | Positive, unidentifed | Negative | 458 |
| NGS-45   | BAL                  | Positive, unidentifed | Negative | 551 |
| NGS-10   | Tissue               | Positive, unidentifed | Negative |                             |
| NGS-34   | Synovial fluid/bone/tissue | Positive, unidentifed | Negative | 11542 |
| NGS-37   | Urethral secretion   | Positive, unidentifed | Negative | 12452 |
| NGS-41   | BAL                  | Positive, unidentifed | Negative | 7502 |
| NGS-47   | BAL                  | Positive, unidentifed | Negative | 3731 |

(Continued)
have been considered clinically-significant and might have improved diagnosis and/or management. In addition, NGS was able to identify possible pathogens in 8/11 Sanger-positive but pathogen-unidentified specimens. These results in a diagnostic advantage to targeted NGS. Moreover, polymicrobial communities identified by NGS may point to particular infection processes that may contribute to patients’ evaluation and optimal management. This is consistent with previous studies that validated the use of NGS for pathogen detection (Culbreath et al., 2019) and compared NGS to culture-based diagnosis (Abayasekara et al., 2017).

**TABLE 1 Continued**

| Sample ID | Material | Sanger results | NGS results | Total number of mapped reads |
|-----------|----------|----------------|-------------|-------------------------------|
| NGS-48    | Tissue   | Positive, unidentified | Polymicrobial (Corynebacterium 7%, Campylobacter ureolyticus 7%, Anaerococcus vaginalis 6%, Finegoldia magna 10%, Peptostreptococcus sp., Peptostreptococcus sp., Peptostreptococcus sp., Streptococcus infantis 1%, H. aegyptius 3%, H. influenza 2%) V1-2 (221); Moraxella catarhalis 6% | 8234 |
| NGS-5     | Tissue   | Positive, unidentified | Polymicrobial (Pseudomonas spp. 31% (P. aeruginosa 13%) Enterobacteriaceae 10% (Salmonella enterica 2.5%)) | 2756 |
| NGS-54    | BAL      | Positive, unidentified | Streptococcus 38% (S. infants 5%) | 1318 |

Each specimen sent to the Clinical Microbiology Laboratory at Rambam for Sanger sequencing, was later sequenced with Ion torrent S5 for the amplification of the V4 hypervariable region of 16s ribosomal gene. Unless noted in the NGS results column, all sequences were found with the primer set V4. Whenever the addition of the variable region V1-2 gave further characterization, it was noted in this table. In the last column, the number of mapped reads with V4. In the NGS results column, the percentage of those reads attributed to each organism.

**Discussion**

The targeted NGS in-house assay was performed on the Ion torrent S5 instrument, used for microbiome purposes, with a predefined threshold of > 1000 mapped reads. The presence of certain pathogens, however, should always be considered as a potential cause of infection, even if the number of reads is below predefined cutoffs, in polymicrobial or nonmonomicrobial results. Such is the case in specimens NGS-44 (699 mapped reads) where *Salmonella* identified by NGS was considered clinically-significant, and NGS-49 (889 reads) where *Proteus mirabilis* among other intestinal pathogens might have been clinically-significant. Conversely, the presence of common commensals should be interpreted carefully. The clinician and the clinical microbiologist must work together to attribute clinical significance to the NGS results.

NGS technology allows for the parallel coverage of all taxa present in a clinical specimen, resulting in the identification of complex microbial communities. The polymicrobial findings in our study likely represented polymicrobial infections. However, alternative explanations should be considered, such as the possibility of commensal microbiota present in non-sterile or sterile body sites, a non-sterile specimen collection technique, or contamination during laboratory workflows. To overcome the latter, species found in negative controls were considered contaminants in our study and their sequences were subtracted from results. During specimen collection and transportation, polymicrobial communities may change in composition. Sanger identifies the best amplified organism which is not necessarily representative of the dominant or pathogenic one.

One central limitation of this study is that only bacterial organisms were targeted (V4 and V1-2 regions of 16S rRNA gene). In addition, samples were selected randomly for this analysis, but not consecutively. The study was non-interventional – NGS results were not used in clinical practice, thus its true effect...
### TABLE 2  Potential added value of NGS over Sanger for patient management.

| Sample ID | Diagnosis                        | Spectrophotometry | NGS results                                                                 | Would have affected diagnosis? | Antimicrobial treatment | Would have changed treatment? |
|-----------|----------------------------------|-------------------|----------------------------------------------------------------------------|-------------------------------|-------------------------|-------------------------------|
| NGS-30    | Chronic internal fixation-associated infection | Negative          | Polymicrobial (*Corynebacterium kroppenstedtii, Enterobacteriaceae, Enterobacter cloacae*) | Yes                           | Cefazolin+ Ciprofloxacin    | Possibly                      |
| NGS-34    | Postpartum sacroiliac joint arthritis | Positive, unidentified | Polymicrobial (*E.coli, Brebundimonas nasuae, Microbacterium cholatatum, Acinetobacter hemolyticus*) | Yes                           | Piperacillin- Tazobactam, subsq. Metronidazole and then Amoxicillin | Yes                           |
| NGS-32    | Necrotizing cervical lymph node (HIV positive) | *Streptococcus sanguis* | Polymicrobial (*Rothia mucilaginosa, Velocinella dispar, Prevotella histicola, Prevotella salivae, Streptococcus australis, Streptococcus infantis, Actinobacillus parahemolyticus, Haemophilus parahemolyticus*) | Yes                           | Unknown (No EMR access)     | Possibly                      |
| NGS-26    | Nosocomial meningitis            | Negative          | Polymicrobial (*Legionella pneumophila, Staphylococcus epidermis*) | Yes                           | Meropenem+ Vancomycin       | Possibly                      |
| NGS-27    | Nosocomial meningitis            | Negative          | *Staphylococcus epidermis* | Yes                           | Meropenem+ Vancomycin       | Possibly                      |
| NGS-23    | Chronic osteomyelitis with Leg abscess | Negative          | *Staphylococcus aureus* | Yes                           | Cefazolin                  | No                            |
| NGS-54    | Cavitary pneumonia (congenital neutropenia) | Positive, unidentified | *Streptococcus salivarius* | Yes                           | Meropenem                  | Possibly                      |
| NGS-44    | Arm fluctuant lesion (AML)       | *Enterobacteriaceae* | *Salmonella, Escherichia* | Yes                           | Amoxicillin- clavulanate    | Possibly                      |
| NGS-25    | Cavitary pneumonia (congenital neutropenia) | *Pseudomonas spp* | Polymicrobial (*Streptococcus salivarius, mitis, anginosus, Prevotella melaninogenica, Velocinella dispar, Pseudomonas aeruginosa*) | No. Positive culture         | Piperacillin- Tazobactam, Subsq. Levofloxacin | No                            |
| NGS-41    | Hilar lymphadenopathy (AML)      | Positive, unidentified | *Pseudomonas aeruginosa* | No. Positive culture          | Levofloxacin               | No                            |
| NGS-4     | Jaw lesion (AML)                 | Negative          | Polymicrobial (*Prevotella oralis, Prevotella oris, Parvimonas micra, Fusobacterium nucleatum, Pseudomonas aeruginosa, P.hibiscicola*) | No.                           | Amphotericin-B + Posaconazole | No                            |

#### Discordant

| Sample ID | Diagnosis                        | Sanger                                                                 | NGS results                                                                 | Would have affected diagnosis? | Antimicrobial treatment | Would have changed treatment? |
|-----------|----------------------------------|------------------------------------------------------------------------|----------------------------------------------------------------------------|-------------------------------|-------------------------|-------------------------------|
| NGS-30    | Chronic internal fixation-associated infection | Negative          | Polymicrobial (*Corynebacterium kroppenstedtii, Enterobacteriaceae, Enterobacter cloacae*) | Yes                           | Cefazolin+ Ciprofloxacin    | Possibly                      |
| NGS-34    | Postpartum sacroiliac joint arthritis | Positive, unidentified | Polymicrobial (*E.coli, Brebundimonas nasuae, Microbacterium cholatatum, Acinetobacter hemolyticus*) | Yes                           | Piperacillin- Tazobactam, subsq. Metronidazole and then Amoxicillin | Yes                           |
| NGS-32    | Necrotizing cervical lymph node (HIV positive) | *Streptococcus sanguis* | Polymicrobial (*Rothia mucilaginosa, Velocinella dispar, Prevotella histicola, Prevotella salivae, Streptococcus australis, Streptococcus infantis, Actinobacillus parahemolyticus, Haemophilus parahemolyticus*) | Yes                           | Unknown (No EMR access)     | Possibly                      |
| NGS-26    | Nosocomial meningitis            | Negative          | Polymicrobial (*Legionella pneumophila, Staphylococcus epidermis*) | Yes                           | Meropenem+ Vancomycin       | Possibly                      |
| NGS-27    | Nosocomial meningitis            | Negative          | *Staphylococcus epidermis* | Yes                           | Meropenem+ Vancomycin       | Possibly                      |
| NGS-23    | Chronic osteomyelitis with Leg abscess | Negative          | *Staphylococcus aureus* | Yes                           | Cefazolin                  | No                            |
| NGS-54    | Cavitary pneumonia (congenital neutropenia) | Positive, unidentified | *Streptococcus salivarius* | Yes                           | Meropenem                  | Possibly                      |
| NGS-44    | Arm fluctuant lesion (AML)       | *Enterobacteriaceae* | *Salmonella, Escherichia* | Yes                           | Amoxicillin- clavulanate    | Possibly                      |
| NGS-25    | Cavitary pneumonia (congenital neutropenia) | *Pseudomonas spp* | Polymicrobial (*Streptococcus salivarius, mitis, anginosus, Prevotella melaninogenica, Velocinella dispar, Pseudomonas aeruginosa*) | No. Positive culture         | Piperacillin- Tazobactam, Subsq. Levofloxacin | No                            |
| NGS-41    | Hilar lymphadenopathy (AML)      | Positive, unidentified | *Pseudomonas aeruginosa* | No. Positive culture          | Levofloxacin               | No                            |
| NGS-4     | Jaw lesion (AML)                 | Negative          | Polymicrobial (*Prevotella oralis, Prevotella oris, Parvimonas micra, Fusobacterium nucleatum, Pseudomonas aeruginosa, P.hibiscicola*) | No.                           | Amphotericin-B + Posaconazole | No                            |

#### Concordant

| Sample ID | Diagnosis                        | Sanger                                                                 | NGS results                                                                 | Would have affected diagnosis? | Antimicrobial treatment | Would have changed treatment? |
|-----------|----------------------------------|------------------------------------------------------------------------|----------------------------------------------------------------------------|-------------------------------|-------------------------|-------------------------------|
| NGS-1     | Leg ulcer                        | *Pseudomonas aeruginosa* | *Pseudomonas aeruginosa* | No                            | No                      | No                            |
| NGS-2     | Left hand abscess                | *Mycobacterium marinum* | *Mycobacterium marinum* | No                            | No                      | No                            |
| NGS-3     | Leg cellulitis                   | *Streptococcus pyogenes* | *Streptococcus pyogenes* | No                            | No                      | No                            |
| NGS-33    | Postpartum sacroiliac joint arthritis | Negative          | *Sneathia sanguinegens* | No.                           | No                      | No                            |
| NGS-21    | Suspected septic arthritis of hip | Negative          | Negative                  | No                            | No                      | No                            |
| NGS-24    | Synovitis                        | Negative          | Negative                  | No                            | No                      | No                            |
| NGS-29    | Nosocomial Meningitis            | Negative          | Negative                  | No                            | No                      | No                            |

(Continued)
on patient management remain unknown. One advantage of this study is that NGS was performed blinded to other microbiological and clinical information.

In conclusion, in this validation study we demonstrated superior pathogen identification with targeted 16S NGS compared to Sanger sequencing in clinical samples. We propose to consider NGS upfront in cases where polymicrobial infections are suspected. Further developments of NGS should include the addition of other important targets such as viral targets or Internal Transcribed Space (ITS) for fungi, as well as antimicrobial resistance genes. To better characterize the accuracy of results, comparison with shotgun metagenomics is necessary.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: [DOI: 10.5281/zenodo.7119981].

Ethics statement

The studies involving human participants were reviewed and approved by Helsinki committee at Rambam HCC, Israel. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Authors’ contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by MPI, BK, and YS. The first draft of the manuscript was written by MPI and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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