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Original Research Article

Diagnostic utility of 16S rRNA meta genomic next-generation sequencing in clinical infectious diseases practice: a retrospective study from South India

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ABSTRACT

Objectives: 16S rRNA sequencing is an emerging diagnostic tool for bacterial syndromes caused by fastidious pathogens and in culture negative infections. However, it can pose significant challenges from pre-analytic to post-analytic phase due to sampling issues, lack of an approved platform and test characteristics. We aimed to evaluate the diagnostic performance of 16S rRNA sequencing in sterile site samples compared to conventional microbiological techniques [CMT] and a composite reference standard [CRS].

Methods: We conducted a retrospective study at a tertiary hospital from January 2022 to July 2024. We included clinical data of patients with sterile site samples processed for both CMT and 16S rRNA sequencing. Sequencing used the Credence Genomics pipeline.

Results: 166 samples met the inclusion criteria: 97 tissue, 35 pus, and 34 fluid samples. Pathogen detection rate was 42.8% [71/166] by CMT and 58.4% [97/166] by 16S rRNA. Concordance between methods was 50.6%. Sensitivity and specificity of 16S rRNA against CMT was 60.6% and 43.2%; and 69.4% and 66.7% against CRS respectively. CMT showed 54% sensitivity and 92.9% specificity against CRS. Combined testing improved sensitivity to 84.7% and accuracy was 78.9%.

Conclusion: 16S rRNA sequencing provides incremental diagnostic sensitivity over conventional microbiological techniques but at the cost of reduced specificity. Its routine frontline use as a standalone diagnostic tool is not supported by our findings. Instead, it may be best reserved for selected culture-negative cases with high clinical suspicion, where results can be interpreted in conjunction with clinical, radiological, and microbiological data by experienced clinicians.

1. Introduction

The diagnostic yield of cultures is affected by pre-analytical factors like prior antibiotic exposure, specimen quality, collection, storage, specimen processing and inability to detect pathogens that grow poorly in culture.

Early reports of 16S ribosomal RNA sequencing showed its ability to classify methanogenic bacterial species [1]. This technique has been used to identify and classify bacteria present in a clinical sample based on genomic registry database. Advantages include a quick turnaround time, the ability to identify non-culturable pathogens, and detection of organisms rendered non-viable by prior antibiotic exposure. However, the major limitation of this method is that it identifies all bacterial DNA in the given sample including contaminants. Most studies have only compared these tests against conventional culture data, which may not be the ideal gold standard.

We aimed to evaluate the diagnostic utility of 16S rRNA sequencing in clinical infectious diseases practice using a commercially available metagenomic next-generation sequencing [m-16S rRNA] platform in sterile site samples. Our objective was to compare the diagnostic accuracy of m-16S rRNA against conventional microbiologic techniques [CMT] and a composite reference standard [CRS].

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2. Methods

2.1. Study design and population

We conducted a retrospective observational study from January 2022 to July 2024 at the Departments of Infectious Diseases and Clinical Microbiology of a tertiary care referral hospital. Retrospective data was collected from electronic medical records [EMR].

2.2. Inclusion criteria

Case records of patients whose clinical samples were processed in the microbiology laboratory for both conventional diagnostics as well as 16S rRNA sequencing were included in the study. Only sterile site samples were included. NGS could have been ordered on the same day of sample retrieval or later. It was performed at an average of 3 days of receiving the sample.

2.3. Exclusion criteria

Samples lacking complete clinical or lab data, unsterile site samples, and those of patients lost to follow-up were excluded.

2.4. Conventional microbiological techniques

Conventional microbiological techniques included gram stain along with bacterial culture with identification by MALDI-TOF and VITEK-MS [Biomerieux], VITEK-2 compact [Biomerieux], or conventional biochemical reactions, and acid-fast bacilli stain along with solid and liquid mycobacterial cultures. Xpert MTB assay [Cepheid, Sunnyvale] was used for *Mycobacterium tuberculosis* detection.

2.5. 16S rRNA sequencing

The 16S rRNA test utilized the Credence Genomics proprietary bioinformatics pipeline using the Oxford Nanopore technology for analysis of clinical isolates [2,3]. Reads obtained from sequencing run were trimmed, and adaptor sequences applied. After trimming, quality control parameters [Phred Quality Score cut off and minimum read length] for all sequence data were checked. The presence or absence of DNA was confirmed through PCR amplification of the bacterial *16S* gene V1-V2 region. Formats generated were mapped to the NCBI-Ref Seq [26:09:2016] database using Credence Infectious Panel Pipeline 1.1.0 [Credence Genomics].

The sequencing and bioinformatics workflow used in this study was based on a previously validated and published pipeline, incorporating established quality control metrics and contamination filtering steps [2]. For reporting, organisms were considered significant only if they constituted ≥10% of total relative abundance within the sample [2]. Organisms were categorized as contaminants as per CDC/NHSN organism list except in special situations like prosthetic joint infections (PJI), implant associated infections, endocarditis etc.

2.6. Composite reference standard

The CRS was defined using a predefined framework incorporating clinical features, imaging findings, histopathology/cytology, microbiological results (including Xpert MTB), and response to therapy. Cases were categorized as bacterial infection, non-bacterial infection, or non-infectious based on predefined criteria [Supplementary Table 1]

In discordant cases, final categorization was based on multidisciplinary review by a team of infectious disease and microbiology experts incorporating longitudinal clinical follow-up.

We excluded 11 samples from data analysis where diagnosis was inconclusive in order to improve diagnostic valve of the study. CRS adjudication was performed retrospectively with no blinding to microbiological results. Blinding was only done at statistician level by labeling the test methods.

2.7. Statistical analysis

We analyzed the sensitivity, specificity, positive and negative predictive value [PPV and NPV], and accuracy of both test methods individually and against the CRS. We used IBM SPSS software to calculate statistical values. The study was approved by the institutional ethics committee [IEC-BMR: AMH-C-S-032/05-24] before commencement.

3. Results

Of a total of 200 samples processed for both cultures and 16S rRNA sequencing during the study period, 166 were included in the final analysis. 34 were excluded due to inappropriate sampling, incomplete data, or invalid CRS [loss to follow-up or inconclusive diagnoses].

Study included 97 [58.4%] tissue samples, 35 [21.1%] pus samples from deep seated abscess and 34 [20.5%] fluid samples. Source and distribution of samples is shown in Fig. 1. Tissue samples from bone/joint including prostheses constituted the majority [74 of 166, 44.5%].

Of the 166 samples, 71 [42.8%] had pathogens detected by CMT and 97 [58.4%] by 16S rRNA sequencing. Composite reference standard was positive for a bacterial infectious syndrome in 124 samples.

3.1. Study analysis in various categories is as described below:

3.1.1. Analysis 1: Concordance/discordance between CMT and 16S rRNA sequencing

We categorized the samples into four categories based on results obtained by CMT and 16S rRNA sequencing and calculated concordance and discordance rates [Supplementary Table 2].

Group 1 included samples which were positive by both CMT and 16S rRNA [n = 53 (32%)].

Group 2 included samples which were positive only by CMT [n = 18 (11%)]. Group 3 included samples where bacteria were solely detected by sequencing [n = 54 (32.4%)]. Group 4 had 41 samples [24.6%] where both the tests did not identify a bacterium.

Group 1 Concordance between the methods was 81.1%[43/53]. Sequencing identified additionally *Bacteroides fragilis* and *Fusobacterium sp.* in two otherwise concordant pus samples. Among the 10 [18.9%] samples with discordant results [Table 1], species level discordance was noted in two *Staphylococcus aureus* samples, which 16S rRNA misidentified as *S epidermidis*. Genus level discordance occurred in 8 samples including one where *Mycobacteroides abscessus* was misidentified as *Klebsiella pneumoniae.*

Group 2 Pathogens missed by sequencing include *M tuberculosis, M abscessus, Burkholderia pseudomallei, S aureus, Pseudomonas aeruginosa, K aerogenes* and *Kytococcus schroeteri.* Pathogens identified in 16samples were clinically compatible based on the CRS**.**This constitutes 9.6% of the study samples where 16S rRNA was negative, and CMT had an impact in treatment decision.

In **Group 3**, 37 of 54 were CRS compatible for a bacterial infectious syndrome, indicating the clinical utility of 16S rRNA over CMT in 22.3% of the cases [supplementary file Table 3].Table 2 depicts the organisms identified in these 37 samples.

Group 4 had 41 samples where both the tests did not identify a pathogen, of which 14 samples had a positive CRS, indicating neither test detected a pathogen in 8.4% of all study samples and in 11.3% of cases with a positive CRS.

Polymicrobial pathogens: Polymicrobial detection was noted in 4 samples by CMT, 16 samples by 16S rRNA of which 12 were compatible with CRS; 2 were CMT discordant and 5 were CMT negative.In samples were polymicrobial reporting was done by 16S rRNA or CMT, sample level analysis was done.

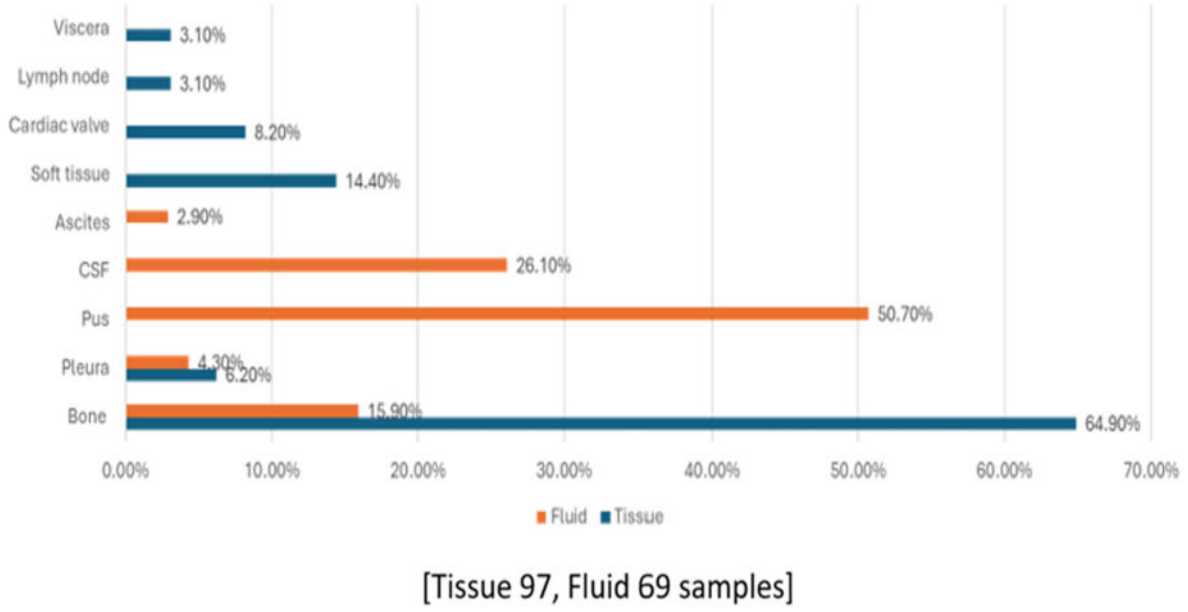

Fig. 1. Source and distribution of study samples.

Table 1
Discordance results between CMT and 16S rRNA
[Genus misidentification n = 10, Species misidentification n = 2].

CMT	16S rRNA identification	CRS
Corynebacterium tuberculostearicum	*Pseudomonas species*	PJI
Staphylococcus aureus	*Staphylococcus epidermidis*	PJI
Staphylococcus aureus	*Staphylococcus epidermidis & Klebsiella pneumoniae*	PJI
Staphylococcus aureus	*Streptococcus agalactiae*	PJI
Pseudomonas aeruginosa	*Enterobacter hormaechei & Cutibacteriam acnes*	PJI
Staphylococcus aureus	*Acinetobacter species*	Osteomyelitis
Mycobacteroides abscessus	*Klebsiella pneumoniae, Acinetobacter*	NTM PJI
Klebsiella pneumoniae	*Cutibacterium acnes*	Skull based osteomyelitis
Staphylococcus epidermidis	*Fusobacterium spp*	Liver abscess
Staphylococcus epidermidis	*Cutibacterium acnes*	Non-infectious syndrome

PJI- Prosthetic joint infection.
NTM- Non tuberculous mycobacteria.

3.1.2. Analysis 2: Comparison of 16S rRNA sequencing vs CMT and CRS in all samples and bone/joint samples

To enhance interpretation and statistical power by minimizing heterogeneity among samples, we analyzed 74 samples specifically obtained from bone and joint sources. Table 3 provides the test characteristics of 16SrRNA sequencing against CMT in all samples and bone/joint samples.

Using the CRS, 42 of 166 samples were classified under non-infectious syndromes or non-bacterial syndromes.For discordant cases, clinical adjudication was done to assign final classification.Table 4 depicts the test characteristics of 16S rRNA against CMT and CRS in all samples.

3.1.3. Analysis 3: Combined CMT and 16S rRNA sequencing against CMT and CRS

The combination of two tests using parallel testing was analyzed. Across all samples, the sensitivity was 84.7% [95% CI: 77.1% - 90.5%], specificity was 61% [95%CI: 45.6% - 76.4%], and accuracy was 78.9% [95% CI: 71.9% - 84.9%]. In bone and joint samples, the sensitivity was

Table 2
Organisms identified solely by 16S rRNA and compatible with CRS.

Serial No.	Sample Site	*Organism[s]*
1	Bone	*Enterobacter species, Cutibacterium species, Staphylococcus aureus*
2	Bone	*Streptococcus agalactiae*
3	Bone	*Staphylococcus epidermidis*
4	Bone	*Mycobacteroides fortuitum*
5	Bone	*Cutibacterium acnes*
6	Bone	*Klebsiella pneumoniae*
7	Bone	*Staphylococcus aureus*
8	Bone	*Streptococcus agalactiae*
9	Bone	*Mycobacteroides abscessus, Acinetobacter baumannii*
10	Bone	*Cutibacterium acnes, Stenotrophomonas maltophilia*
11	Bone [Prosthesis]	*Streptococcus pneumoniae*
12	Bone [Synovial fluid]	*Streptococcus dysgalactiae*
13	Bone [Synovium]	*Cutibacterium acnes*
14	Bone [Synovium]	*Pseudomonas aeruginosa*
15	Cardiac [valve]	*Staphylococcus aureus*
16	Cardiac [valve]	*Streptococcus agalactiae*
17	Cardiac [valve]	*Corynebacterium striatum*
18	CSF	*Streptococcus pneumoniae*
19	Pleural	*Streptococcus anginosus group*
20	Pleural	*Streptococcus salivarius, Streptococcus thermophilus*
21	Pleural	*Streptococcus pneumoniae*
22	Pleural	*Fusobacterium nucleatum*
23	Pleural	*Fusobacterium spp*
24	Pus	*Burkholderia pseudomallei*
25	Pus	*Streptococcus pyogenes*
26	Pus	*Streptococcus pyogenes*
27	Pus	*Mycobacteroides abscessus*
28	Pus	*Mycobacteroides abscessus*
29	Pus	*Mycobacteroides abscessus*
30	Pus	*Bartonella species*
31	Pus	*Escherichia coli*
32	Pus [IC][a]	*Streptococcus intermedius*
33	Soft tissue	*Streptococcus pyogenes*
34	Soft tissue	*Mycobacteroides abscessus*
35	Soft tissue	*Nocardia species*
36	Soft tissue	*Bartonella species*
37	Viscera	*Mycobacterium tuberculosis, Pseudomonas aeruginosa*

[a] IC – Intracranial.

Table 3
16S rRNA and CMT against CRS in all samples and bone/joint samples.

Groups	All samples [N = 166]		Bone/joint samples [N = 74]	
	CMT	16S rRNA sequencing	CMT	16S rRNA sequencing
Sensitivity% [95% CI]	54.0% [44.9-63]	69.4% [60.4 - 77.3]	61.4% [47.6-74]	63.2% [49.3 - 75.6]
Specificity % [95% CI]	92.9% [80.5 - 98.5]	66.7% [50.5-80.4]	94.1% [71.3-99.9]	70.6% [44-89.7]

Table 4
16S rRNA against CMT and clinical correlation (Composite reference standard).

CMT			CRS		
	All samples	**[N = 166]**		**All samples**	**[N = 166]**
16S rRNA	Positive	Negative	**16S rRNA**	Positive	Negative
Positive	43	54	Positive	86	14
Negative	28	41	Negative	38	28
	Test performance [CI]			Test performance [CI]	
Sensitivity	60.6% [48.3% - 71.9%]		Sensitivity	69.4% [60.4% - 77.3%]	
Specificity	43.2% [33.0% - 53.7%]		Specificity	66.7% [50.5% - 80.4%]	

85.9%, specificity was 64.45%, and accuracy was 81.1% [Supplementary Table 4].

4. Discussion

16S rRNA sequencing has been evaluated against CMT in various clinical syndromes such as respiratory infections, urinary tract infections, prosthetic joint infections and infective endocarditis [3–8]. Our clinical study is the largest to date from India that compares 16S rRNA metagenomics next generation sequencing against both CMT and CRS.

4.1. 16S rRNA sequencing and CMT

Our principal findings include that when comparing 16S rRNA sequencing against CMT, the overall sensitivity and specificity of 16S rRNA was 60.6% and 43.2% respectively. This was almost similar for bone/joint samples as well [Table 4]. 16S rRNA identified a pathogen in 18% more samples than CMT, and concordance [positive or negative] between the tests was only 50.6%. In an earlier lab-based study using the same technique of sequencing [Credence RID pipeline], specificity was found to be 52.7% against culture in 97 samples, though the sensitivity was higher 91.8% [2]. A real-time prospective study with the same pipeline on ICU patient sterile site samples including blood showed a better sensitivity of 94.1% and specificity of 86.6%, though the exact incremental value of sequencing over culture was not firmly established [9].

4.2. Critical factors in 16S rRNA sequencing

Advantage of 16S rRNA was its ability to detect difficult to culture organisms like NTM, *Bartonella* etc. However, it failed to detect common pathogens like *S aureus, S epidermidis, P aeruginosa, K aerogenes, M tuberculosis* and *B pseudomallei* up to genus/species level, possibly because the diagnostic performance of 16S rRNA sequencing is influenced by several pre-analytical and analytical factors.

Despite inclusion in reference databases, certain clinically important pathogens such as *Mycobacterium tuberculosis* complex and *Burkholderia pseudomallei* may be missed or misidentified by 16S rRNA sequencing. Reasons include low organism burden, as in partially treated infections, as well as primer bias affecting amplification efficiency of specific taxa. In addition, stringent bioinformatic thresholds for read mapping and relative abundance may lead to exclusion of low-level but clinically significant organisms. Hence, cautious interpretation is warranted as per the clinical context.

In TB-endemic settings, the sensitivity of 16S-based assays for mycobacteria is limited due to low bacillary load, slow growth characteristics, and sub-optimal primer coverage. Therefore, 16S rRNA sequencing should not be used as a substitute for established diagnostic methods for tuberculosis.

4.3. 16S rRNA sequencing and CRS

Against CRS, 16S rRNA sequencing had higher sensitivity [69.4% Vs 54%], and lower specificity [66.7% VS 92.9%] than CMT. However, despite exclusion of unsterile site samples, the results had no correlation with CRS in 31% and it over-identified pathogens in 14 samples classified under non-bacterial infection syndromes by CRS. Pathogens identified by 16S rRNA found to be clinically incompatible were *Acinetobacter baumannii*, *Pseudomonas species*, *Mobiluncus spp*., *Lactobacillus spp., B cepacia complex, C acnes, S epidermidis, K pneumoniae, and Paracoccus species*. False positive results are frequently attributable to background DNA contamination introduced during sample collection, transport, or laboratory processing, as well as accumulation of environmental or reagent-derived DNA during sequencing workflows [10].

In samples where CRS was assigned bacterial osteomyelitis/PJI, *Cutibacterium acnes* was considered a true pathogen.

4.4. Literature comparison

The low specificity is currently a major deterrent as it could lead to potential misdiagnoses and antibiotic overuse.In the largest prospective study by Rampini et al., the sensitivity of broad-range PCR was 81.9% and the specificity 93.8% against culture [11]. Concordance of culture and PCR was found in 90.4%. For culture negative infections, they showed a sensitivity of 42%, specificity of 100%, PPV 100% and NPV of 80.2%. In our retrospective study, we found the concordance only in 50.1% and discordance in 11.5% of culture-positive 16S rRNA-negative and 32% culture-negative 16S rRNA-positive samples. On several occasions, NGS had been requested after the samples had been processed for culture, indicating the possibility extraneous DNA contamination. This is reflective of real-world practice, especially in developing countries, where NGS is not ordered upfront.

In another study comparing utility of 16S rRNA [Nanopore 16S sequencing] and culture, environmental bacteria such as *Pelomonas saccharophila*, *Cutibacterium acnes*, and *Stenotrophomonas rhizophila* were detected. The overall concordance ranged from 63.4% to 87.4% according to the 16S rRNA pipeline used – nano CLUST, Epi2me, Emu [3].

Cutibacterium acnes were detected only by 16S rRNA in 8 samples, out of which 3 were osteomyelitis and were considered compatible with CRS. This detection by 16S rRNA is however in contrast with a prior study which concluded that next-generation sequencing requires a higher inoculum compared to conventional anaerobic culture [12].

4.5. Additive analysis

To assess the ***incremental* effect** of 16S rRNA performed on samples where conventional cultures were negative, parallel additive testing was analyzed. The overall sensitivity increased to 84.7% and specificity dropped to 61% when compared to stand alone CMT. This indicates the incremental role of the test to CMT alone.

4.6. Bone and joint samples

By analyzing the ***bone and joint samples*** [n = 74], we found only a slight difference in sensitivity between NGS and CMT [63.2% VS 61.4%]. Specificity of 16S rRNA was lower than CMT [70.6% VS 94.1%] but greater when compared to specificity in the overall samples [70.6% Vs 61%]. Our study included synovial aspirates, operative samples from PJI and osteomyelitis, and image guided biopsies. Among the 14 PJI in our study, CMT was positive in 13, and 16S rRNA positive in 10 samples. Ina meta-analysis of NGS in peri-prosthetic infections, NGS showed higher sensitivity [80.6% to 95.9%] and specificity [84.6% to 95.2%] [7]. In a retrospective study on m16S rRNA in spinal infections correlating with the histopathology, the sensitivity of m16S rRNA were significantly higher than those of the culture group [82.3% versus 17.5%], whereas the specificity of was lower [42.9% vs 76.9%]. We found similar results but higher specificity than what was reported by Hui Lv et al. [13].

4.7. CNS samples

In our study, the CRS positive ***CNS infection*** samples were only 4 of 18, and we could not assess its utility. The 16S rRNA method positively impacted one patient outcome by identifying a *Streptococcus pneumoniae*but falsely identified bacteria in two CSF samples [*Streptococcus pyogenes, Klebsiella pneumoniae* with *Mycoplasma*] with negative CRS. 16S rRNA failed to detect one *Mycobacterium tuberculosis,* identified by Xpert MTB Ultra assay.

In a prospective study on CNS infections based on CSF analysis, 16S rRNA assay identified more potential pathogens than conventional testing [32 vs 27]. Of these, 8 of the 13 diagnoses had a clinical effect, where neurologic infections remain undiagnosed [14].

4.8. Cardiac-valve tissue samples

Among samples from ***cardiac valve tissue***, in which 6 were compatible with infective endocarditis, 16S rRNA identified a relevant pathogen in 3 cases which were missed by CMT. Both tests were negative in a sample fitting into IE. In a study evaluating 40 cases of IE, 16S rRNA detected pathogen in 28 samples, sensitivity of 16S rRNA sequencing and cultures on valve were 75% and 55%, respectively. In patients with antibiotic exposure, positivity rates of blood cultures and 16S rRNA sequencing of valves were 11% and 76%, respectively [8].

4.9. Limitations

Our study has several limitations including retrospective analysis, inclusion of heterogenous study samples, and timing snd selective ordering of NGS at physician discretion, lack of blinding.

However, our study reflects a real-world situation with inputs from clinical microbiologist and infectious diseases physicians, making the results applicable to clinical practice.

5. Conclusion

In our retrospective analysis, we found a good sensitivity but low specificity and negative predictive value for 16SrRNA sequencing. The technique missed important pathogens like *Mycobacterium tuberculosis, Burkholderia pseudomallei, Staphylococcus aureus* etc.Stringent precautions during collection, storage and processing of sterile specimens are required to exclude potential contaminants. It was a useful adjunct in specific cases, especially in culture negative cases with fastidious pathogens, when interpreted in the right clinical context.

Large scale prospective multicentric studies evaluating a single well-designed platform in specific clinical syndromes, addressing the issues noted with both sensitivity and specificity, are needed for the test to get regulatory approval, and to become standard of care. In countries like India, where multiple NGS platforms are commercially available, it is important to use 16S rRNA sequencing in the right clinical context in conjunction with conventional microbiologic techniques. Involvement of both clinical microbiologist and infectious diseases physician is vital for diagnostic and antibiotic stewardship.

Ethical approval

Institutional ethics committee approval was issued on 5.6.2024 with reference number AMH-C-S-032/05-24.

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CRediT authorship contribution statement

Pugazhvanan CR: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. **Vidya Krishna:** Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Nandini Sethuraman:** Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing. **Senthur Nambi P:** Methodology, Supervision, Writing – review & editing. **Venkatasubramanian Ramasubramanian:** Methodology, Supervision, Writing – review & editing. **Praveen Balaguru:** Methodology, Writing – review & editing. **Ram Gopalakrishnan:** Conceptualization, Methodology, Supervision, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nandini Sethuraman reports a relationship with Microgenomics India Private Ltd that includes: consulting or advisory. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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