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Diagnostic Value of 16S Ribosomal RNA Gene Polymerase Chain Reaction/Sanger Sequencing in Clinical Practice

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Background. Accurate microbiologic diagnosis is important for appropriate management of infectious diseases. Sequencing-based molecular diagnostics are increasingly used for precision diagnosis of infections. However, their clinical utility is unclear.

Methods. We conducted a retrospective analysis of specimens that underwent 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) followed by Sanger sequencing at our institution from April 2017 through March 2019.

Results. A total of 566 specimens obtained from 460 patients were studied. Patients were considered clinically infected or noninfected based on final diagnosis and management. In 17% of patients, 16S rRNA PCR/sequencing was positive and in 5% of patients, this test led to an impact on clinical care. In comparison, bacterial cultures were positive in 21% of patients. Specimens with a positive Gram stain had 12 times greater odds of having a positive molecular result than those with a negative Gram stain (95% confidence interval for odds ratio, 5.2–31.4). Overall, PCR positivity was higher in cardiovascular specimens (37%) obtained from clinically infected patients, with bacterial cultures being more likely to be positive for musculoskeletal specimens ($P < .001$). 16S rRNA PCR/sequencing identified a probable pathogen in 10% culture-negative specimens.

Conclusion. 16S rRNA PCR/sequencing can play a role in the diagnostic evaluation of patients with culture-negative infections, especially those with cardiovascular infections.

Keywords. molecular diagnostics; 16S rRNA gene PCR; Sanger sequencing; broad range bacterial PCR; bacterial infections.

Accurate identification of microbial pathogens informs targeted therapy, and ultimately increases the likelihood of favorable clinical outcomes [1]. Standard identification of bacteria in clinical specimens involves Gram stain, followed by growth of organisms using appropriate culture media. Despite using standard microbiologic practices and testing, in patients with high underlying clinical suspicion of infection, cultures often remain negative. This may be related to prior antimicrobial therapy or the inability of fastidious organisms to grow on standard culture media [2–5]. Lack of microbiologic diagnosis in patients with a high index of suspicion for infection often leads to use of broad-spectrum antimicrobial therapy, which results in increased risk of selection for antimicrobial resistance and other antibiotic-associated side effects and does not invariably "cover" the pathogen present [6–8].

In the last decade, sequencing-based molecular diagnostics have been increasingly used in complex cases to overcome limitations of culture-based diagnostics. One such tool, based on amplification and sequencing of the 16S ribosomal RNA (rRNA) gene, is becoming more widely available, with improvements in turnaround time, over the last few years. The 16S rRNA gene, present in all bacteria, has variable and conserved regions that provide for identification of most bacteria to the genus or species level, depending on the design of the assay. Species-level identification has been reported to be achievable in 65%–91% of cases in the literature, depending on the region used for sequencing [9–11]. 16S rRNA gene polymerase chain reaction (PCR) followed by Sanger sequencing (16S rRNA PCR/sequencing) was initially used in clinical laboratories to identify isolates not easily identifiable by phenotypic means [12]. More recently, 16S rRNA PCR/sequencing is being used directly on clinical specimens, especially to identify difficult-to-cultivate bacteria or those rendered noncultivatable by antimicrobial therapy [13–15].

Studies comparing 16S rRNA PCR/sequencing with culture-based diagnostics have reported low sensitivity and specificity [16–18]. Despite known limitations of 16S rRNA PCR/sequencing, there is growing interest in evaluating its clinical use in culture-negative infections [19–22]. Few studies have

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examined the clinical utility and impact of 16S rRNA PCR/sequencing on patient management. Given the cost and technical complexity involved, attempts have been made to target use of 16S rRNA PCR/sequencing to patients who are most likely to benefit from it to maximize diagnostic yield and cost effectiveness. The aim of the current study was to assess real-world performance of 16S rRNA PCR/sequencing, especially in culture-negative infected patients and to identify specimen and patient factors associated with the highest yield, as well as to assess the impact of 16S rRNA PCR/sequencing on clinical management.

METHODS

We performed a retrospective chart review of patients whose specimens underwent 16S rRNA PCR/sequencing testing at our institution from April 2017 to March 2019. These were identified by searching a microbiologic database. All patients whose specimens underwent 16S rRNA PCR/sequencing regardless of final diagnosis were included, including those with fungal or viral infection. Demographic, clinical, microbiologic and histopathologic data were extracted by review of the electronic medical record and collected in a REDCap database. The study was approved by the Mayo Clinic Institutional Review Board. If multiple specimens were collected from the same patient during a single procedure, specimens with the same source and the same 16S rRNA PCR/sequencing result were counted as a single specimen for the purposes of analysis. There was no restriction on ordering 16S rRNA PCR/sequencing in place at the time of this study, aside from an option to send a specimen to the laboratory to hold for 16S rRNA PCR/sequencing to be performed in the 2 weeks after specimen receipt if that testing were to be ordered in that time frame.

Definitions

Synovial fluid, pleural fluid, pericardial fluid, and abscess aspirates were considered "fluid" specimens, with other specimens (eg, valve and other surgical specimens) considered "tissue" specimens. Patients were classified as having clinical infection if a final diagnosis by the treatment team was deemed infectious and there was resolution of symptoms with antimicrobial therapy. Patients were classified as having "no infection" if the final diagnosis was not infection and they did not receive antimicrobial therapy (beyond initial empiric therapy). Prior antibacterial therapy was defined as any antimicrobial therapy administered in the 2 weeks preceding 16S rRNA PCR/sequencing. 16S rRNA PCR/sequencing was deemed to have an "effect on clinical management" if the result led to escalation or de-escalation, or initiation or discontinuation, of antimicrobial therapy.

Sample Processing Methods

Sample processing and cultures were conducted in the Initial Processing and Clinical Bacteriology Laboratories of the Division of Clinical Microbiology, Mayo Clinic, Minnesota. Isolated bacteria were identified using conventional biochemical identification and/or matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. 16S rRNA PCR/sequencing and sequencing was performed in the Mayo Clinic Bacteriology Laboratory, as described elsewhere [23]. Briefly, specimens were processed by incubation with proteinase K followed by lysis through rapid shaking with silica/zirconium beads at 100°C. Nucleic acid extraction was performed manually using Zymo Genomic DNA Clean & Concentrator-10. PCR was performed on a LightCycler 480II instrument (Roche Diagnostics) and used to amplify approximately 530 base pairs of the bacterial 16S rRNA gene (V3–V4) with SYBR Green detection of amplified product.

The following primers were used: forward, 5′-CGGCCCAG ACTCCTACGGGAGGCAGCA-3′; reverse, 5′-GCGTGGAC TACCAGGGTATCTAATCC-3′. Five microliters of extracted DNA was added to the reaction mixture that contained 0.08 µL of each primer, 10 µL of LightCycler 480 SYBR green I Master, 0.5 µL of double-stranded DNase, 0.5 µL of dithiothreitol, and 3.84 µL of sterile water. Amplification inhibition was detected by means of a second PCR reaction performed using the extracted specimen spiked with a low concentration of positive control DNA. Samples with cycle threshold values ≤32 cycles underwent bidirectional Sanger sequencing using an Applied Biosystems 3500xL instrument. Consensus sequences of ≥400 base pairs were used for identification.

Statistical Analysis

Student *t* and Mann-Whitney tests were used to compare means and medians for continuous variables with χ^2 tests used for independent proportions. McNemar's test was used to calculate sensitivity and specificity, where applicable, and of confidence intervals were calculated using the binomial exact method. Differences were considered statistically significant at $P < .05$. Statistical analyses were performed using JMP 15.2.1 software.

RESULTS

A total of 566 specimens that underwent 16S rRNA PCR/sequencing testing were identified from 460 patients during the study period (April 2017 to March 2019). The median patient age was 63 years (interquartile range, 2–93 years); 46% of patients were female. Comorbid conditions included diabetes mellitus (18%), solid cancer (10%), hematologic cancer (8%), hematopoietic stem cell transplantation (3%), solid organ transplantation (5%), human immunodeficiency virus infection (1%), and other causes of immunosuppression (19%). The most common specimen source was the musculoskeletal (MSK) system (69%), followed by the central nervous system (8%),

and cardiovascular system (7%), and skin and soft tissues (4%). Of the specimens, 56% were tissues and 44% fluids.

The overall 16S rRNA PCR/sequencing positivity rate was 17.1% (97 of 566), with 90% (88 of 97) of positive results being from patients with clinical infections (Table 1). DNA amplification was not achieved in 2% of specimens (10 of 566) owing to the presence of inhibitors in the sample. In 22% of specimens with positive results (n = 21), the bacterial 16S rRNA gene was detected by amplification; however an organism could not be identified by Sanger sequencing, for the most part owing to mixed sequences.

Bacterial cultures of 20% of specimens (114 of 566) were positive; 87% of culture-positive specimens (99 of 114) were from patients with clinical infection. The overall concordance between PCR and culture was 77% (429 of 556) (Table 1). The sensitivity and specificity of 16S rRNA PCR/sequencing, considering clinical diagnosis as the reference standard, were 30% and 97%, respectively. In comparison, the sensitivity and specificity of bacterial cultures were 34% and 94%, respectively.

Among the 54% of specimens (303 of 566) from patients with a final diagnosis of infection, bacterial cultures were positive in

Table 1. Characteristics of Specimens and Comparison Between Infected and Noninfected Groups

	Specimens, No. (%)[a]			
Characteristics	All specimens (N = 566)	Infection (n = 303)	No Infection (n = 263)	*P* Value
ESR, mean, mm/h	36	42	29	<.001
CRP, mean, mg/L	44	53.9	31.8	<.001
Specimen type				
Fluid	252 (44)	95 (31)	157 (60)	…
Tissue	314 (56)	208 (69)	106 (40)	
Type of specimen				
Fresh	524 (93)	279 (92)	245 (93)	…
FFPE	35 (6)	20 (7)	15 (6)	
Unknown	5 (1)	3 (1)	2 (1)	
Gram stain (n = 536)				
Positive	24 (4.5)	22/282 (7.8)	2/254 (0.8)	<.001
Negative	512 (95.5)	260/282 (92)	252/254 (99)	
Bacterial culture				
Positive	114 (20)	99 (33)	15 (6)	…
Negative	437 (77)	194 (64)	243 (92)	
Not performed	15 (2.6)	10 (3)	5 (2)	
Culture concordance with PCR[b]	429/556 (77)	198/298 (66.4)	231/258 (89.5)	<.001
PCR positive				
Positive with specific ID	76 (13)	72 (23.7)	4 (1.5)	<.001
DNA detected, no ID	21 (4)	16 (5.3)	5 (1.9)	
Total	97 (17.5)	88 (29)	9 (3.4)	
PCR negative	459 (81)	210 (69.3)	249 (94.6)	<.001
PCR inhibitor present	10 (1.7)	5 (1.6)	5 (1.9)	
Pathology performed				
Yes	249 (44)	157 (51.8)	92 (35)	…
No	312 (55)	141 (46.5)	171 (65)	
Specimen source				
MSK	391(69)	206 (68)	185 (70.3)	…
Cardiovascular	41 (7)	27 (9)	14 (5)	
CNS	44 (7.2)	19 (6)	25 (9.5)	
Skin or soft tissue	23 (4)	15 (5)	8 (3)	
Other	67 (12)	36 (12)	31 (12)	
Prior antibiotics	182 (32)	146 (48)	36 (13.7)	<.001
Impact on clinical care	31 (5)	25 (8)	6 (2)	
Changes in therapy	(n = 31)	(n = 25)	(n = 6)	.002
Escalation	6 (1)	6 (2)	0	
De-escalation	14 (2.5)	13 (4.2)	1 (0.3)	
Initiation	6 (1)	6 (2)	0	
Discontinuation	5 (0.8)	0	5 (2)	

Abbreviations: CNS, central nervous system; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; FFPE, formalin-fixed paraffin-embedded; ID, identification; MSK, musculoskeletal; PCR, polymerase chain reaction.

[a]Data represent no. (%) of specimens unless otherwise specified.

[b]Positive and negative concordance.

33% (99 of 303). In 18% of patients (53 of 303), both bacterial cultures and PCR were positive (Figure 1). There was concordance between culture and PCR in 74% (39 of 53), whereas in 26% (14 of 53), PCR was either discordant (n = 4) or detected bacterial DNA only without identification of a particular organism owing to mixed sequences (n = 10) (Supplementary Table 1).

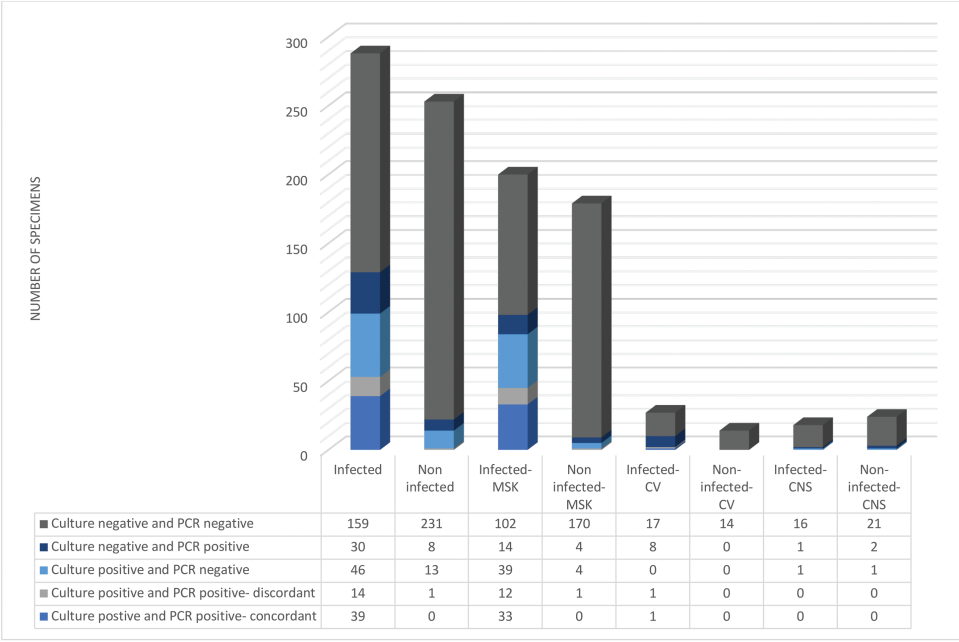
In 15% of clinically infected patients (46 of 303), 16S rRNA PCR was negative despite there being positive cultures. In almost half of these patients, cultures were positive for coagulase-negative *Staphylococcus, Micrococcus,* or *Corynebacterium* species or there was polymicrobial growth (Supplementary Table 2).

In 10% of patients (30 of 303) with clinical infection, 16S rRNA PCR/sequencing was positive, whereas bacterial cultures were negative. In 5 of these patients, only bacterial DNA was detected but a particular organism could not be identified owing to mixed sequences (Supplementary Table 3). In 10 patients with clinical infection, cultures were not obtained; 16S rRNA PCR/sequencing was positive in 5 (Supplementary Table 3). In 53% of infected patients (159 of 303), both bacterial cultures and 16S rRNA PCR were negative; in 12 of these, a fungal organism was identified by culture.

Results of 16S rRNA PCR/sequencing were positive in 4 noninfected patients. Bacteria identified in these noninfected patients included *Lactobacillus* species, and *Streptococcus* species in 1 patient each, and *Staphylococcus* species in 2 patients. Bacterial cultures were positive in 15 patients without clinical infection, with cultures yielding *Cutibacterium acnes* in 6, *Micrococcus* species in 1, coagulase-negative *Staphylococcus* species in 5, and polymicrobial growth in 3.

Inflammatory markers, including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were higher in infected versus noninfected patients overall (both $P < .001$). Similarly, patients with positive 16S rRNA PCR/sequencing results had higher mean CRP and ESR values than those with negative 16S rRNA PCR results (both $P < .001$). In the clinically infected subgroup, there was a difference in CRP levels between patients with positive versus negative 16S rRNA PCR/sequencing results ($P = .005$); however, ESR values did not differ ($P = .12$). Specimens with positive Gram stains obtained in the clinical microbiology laboratory had a 12 times greater odds of positive 16S rRNA PCR/sequencing results than those with negative Gram stains (95% confidence interval for odds ratio, 5.2–31.4).

Overall, although more tissue than fluid specimens were collected from clinically infected versus noninfected patients ($P < .001$) (Table 1), there was no difference in PCR positivity between fluid and tissue specimens ($P = .8$) (Table 2). Subgroup analysis of the clinically infected group showed that 16S rRNA PCR/sequencing positivity was higher for fluid than for tissue specimens (40 vs 25%; $P = .009$). Six percent of samples were formalin-fixed paraffin-embedded (FFPE) tissues; although there was no difference in PCR positivity rates between FFPE and fresh tissue specimens within the clinically infected



Specimens with PCR inhibitors (n=10) and specimens without bacterial cultures (n=15) are not included

Figure 1. Culture and polymerase chain reaction (PCR) results by clinical diagnosis. Specimens with PCR inhibitors (n = 10) and specimens without bacterial cultures (n = 15) are not included. Abbreviations: CNS, central nervous system; CV, cardiovascular; MSK, musculoskeletal.

Table 2. Characteristics of Polymerase Chain Reaction (PCR)–Positive Versus PCR-Negative Specimens

Characteristic	Specimens, No. (%)[a]		
	PCR Positive (n = 97)	PCR Negative (n = 459)	*P* Value
ESR, mean, mm/h	46	34	<.001
CRP, mean, mg/L	74	37	<.001
Specimen source			
MSK	65 (17)	319 (83)	.21
Cardiovascular	10 (24)	31 (76)	
CNS	4 (9)	40 (91)	
Skin or soft tissue	6 (27)	16 (73)	
Other	12 (18)	53 (82)	
Specimen type			
Fluid	42 (43)	206 (45)	.8
Tissue	55 (57	253 (55)	
Specimen			
Fresh	91 (94)	424 (93)	.6
FFPE	4 (4.17)	30 (7)	
Unknown	2 (2)	5 (1)	
Gram stain (n = 536)			
Positive	16 (18)	8 (2)	<.001
Negative	70 (81)	432 (98)	
Bacterial cultures			
Positive	54 (59)	59 (13)	<.001
Negative	38 (41)	390 (87)	
Final microbiologic diagnosis			
Monomicrobial	67 (69)	55 (12)	…
Polymicrobial	16 (17)	10 (2)	
Mycobacterial	1 (1)	16 (4)	
Fungal	0	12 (3)	
Microbiology undefined	4 (4)	117 (25)	
Noninfectious	9 (9)	249 (54)	
Infectious syndrome	88 (91)	210 (46)	<.001
Prior antimicrobial therapy	39 (40)	139 (30)	.07
Impact on clinical care	23 (24)	8 (1.7)	<.001
Changes in therapy			
Escalation	6 (6)	0	…
De-escalation	11 (11)	3 (0.6)	
Initiation	6 (6)	0	
Discontinuation	0	5 (1)	

Abbreviations: CNS, central nervous system; CRP, C-reactive protein; CSF, cerebrospinal fluid; ESR, erythrocyte sedimentation rate; FFPE, formalin-fixed paraffin-embedded; MSK, musculoskeletal; PCR, polymerase chain reaction.

[a]Data represent no. (%) of patients unless otherwise specified.

group, fresh specimens were more likely to be PCR positive than specimens overall (18% vs 12%; $P = .04$).

Overall, 16S rRNA PCR/sequencing was more likely to be positive with cardiovascular specimens (10 of 27 [37%]) than with other specimen sources (50 of 175 [28%]) in clinically infected patients (Table 3). In contrast, rates of Gram stain positivity (4%) and bacterial culture positivity (7%) were much lower with cardiovascular specimens than with other specimen types. Among clinically infected patients, cardiovascular specimens were less likely to be culture positive than other specimen sources ($P = .002$). In contrast, bacterial cultures were more likely to be positive from MSK compared with non-MSK specimens ($P < .001$). Most central nervous system specimens were cerebrospinal fluid (CSF) (77%); 16S rRNA PCR/sequencing positivity was higher for brain biopsy specimens than for CSF specimens (3 vs 1 specimen; $P = .01$). Two of 3 brain biopsy results led to a change in clinical management.

Overall, 32% of patients had received antimicrobial therapy in the 2 weeks preceding testing by 16S rRNA PCR/sequencing. More infected compared with uninfected patients received antecedent antimicrobial therapy (48 vs 14%; $P < .001$). There was no difference in receipt of antecedent antimicrobial therapy with regard to PCR or culture positivity overall. 16S rRNA PCR/sequencing results led to a change in clinical management in 31 patients (5% overall), including escalation in therapy in 6, de-escalation in 14, initiation in 6, and discontinuation in 5. Positive 16S rRNA PCR/sequencing results were more likely than negative results to lead to clinical management changes ($P < .001$).

DISCUSSION

In this study, 16S rRNA PCR/sequencing identified a potentially pathogenic organism in 14% of clinically infected patients who either had negative cultures or did not have cultures performed. Among infected patients, the 16S rRNA PCR/sequencing positivity rate was higher for cardiovascular specimens than for other specimen types. Bacterial culture positivity rates were highest in MSK specimens.

In several cases, 16S rRNA PCR/sequencing identified a difficult to culture or uncultivable organism, including *Ureaplasma* species, *Mycoplasma hominis*, *Lawsonella clevelandensis*, *Treponema* species, and *Mycobacterium lepromatosis* (single cases of each). In the *M. lepromatosis* case, which has been described elsewhere, acid-fast bacilli were seen in skin biopsy and the clinical picture matched the 16S rRNA PCR/sequencing result [23]. The patient had clinical improvement after receiving treatment directed against *M. lepromatosis*. In a case of *M. hominis* endocarditis, pathogen-directed therapy led to resolution of infection without recurrence. Similarly, in the *Ureaplasma* native joint septic arthritis case, *Ureaplasma urealyticum*–specific PCR results were positive and there was clinical improvement after treatment with azithromycin and doxycycline. Likewise, in the syphilitic panuveitis case, the rapid plasma reagin titer was 1:1024. *L. clevelandensis,* which was identified in a case of endovascular graft infection, is increasingly recognized as a cause of purulent infections. It is a slow-growing, anaerobic gram-variable branching rod that requires incubation beyond the usual standard number of days of incubation and hence may be missed by standard cultures [24].

Previous studies and our unpublished experience have demonstrated identification by 16S rRNA PCR/sequencing of other

Table 3. Characteristics and Sources of Musculoskeletal, Cardiovascular, and Central Nervous System Specimens

Characteristic	Musculoskeletal (n = 391)	Cardiovascular (n = 41)	CNS (n = 44)
Specimen source	Synovial fluid, 50%; periprosthetic joint tissue, 18%; native joint tissue, 8%; bone 4%; spine, 10%; hardware sonicate fluid, 2%; other, 9%	Heart valve, 66%; aortic graft, 15%; other, 20%	CSF, 77%; brain biopsy, 23%
Specimen type, (%)			
Fluid	50	5[a]	23[a]
Tissue	50	95	77
Gram stain positivity rate, no. positive/total (%)			
Infected	17/195 (9)	1/27 (4)	1/17 (6)
Uninfected	1/182 (0.5)	0/13 (0)	0/23 (0)
Total	18/377 (5)	1/40 (2.5)	2.5 (3)
Clinically diagnosed infectious syndrome, (%)	52	66	43
Culture positivity rate, no. positive/total (%)			
Infected	84/204 (41)	2/27 (7)	1/18 (5.5)
Uninfected	5/182 (3)	0/14 (0)	1/24 (4)
Total	89/386 (23)[a]	2/41 (5)	2/42 (5)
PCR positivity rate, no. positive/total (%)			
Infected	60/202 (30)	10/27 (37)	2/19 (10.5)
Uninfected	5/182 (3)	0/14 (0)	2/25 (8)
Total	65/384 (17)	10/41 (24)	4/44 (9)
Prior antibiotics, no./total (%)			
Infected	88/206 (43)	19/27 (70)	15/19 (80)
Uninfected	21/185 (11)	5/14 (36)	3/25 (12)
Total	109/391(28)	24/41 (59)	18/44 (41)
Impact on clinical care, no./total (%)			
Infected	12/205 (6)	7/27 (26)	2/19 (10.5)
Uninfected	4/185 (2)	2/14 (14)	0/25 (0)
Total	16/390 (4)	9/41 (22)[a]	2/44 (5)

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; PCR, polymerase chain reaction.

[a] $P < .05$

organisms that are challenging to cultivate, including *Bartonella* species, *Tropheryma whipplei*, and *Coxiella burnetti* [25, 26]. In clinically infected cases with positive cultures and negative 16S rRNA PCR results, approximately 50% had coagulase-negative *Staphylococcus* species or *Micrococcus* or *Corynebacterium* species identified, which may represent contaminants rather than pathogens.

Although more clinically infected compared with noninfected patients had received antibacterial therapy before 16S rRNA PCR/sequencing, treatment did not obviously affect rates of 16S rRNA PCR/sequencing or culture positivity. The decision to administer empiric therapy is usually dictated by the clinical picture; in patients with a final diagnosis of an infectious syndrome, a high suspicion of infection at presentation may have prompted initiation of empiric therapy.

The diagnostic yield of 16S rRNA PCR/sequencing has been variable in the literature and may depend on patient and specimen as well as assay characteristics. Previous studies have suggested that 16S rRNA PCR/sequencing on tissue-based specimens may be more likely to yield a clinically significant result than fluid samples [27]; however, in this cohort, fluid type specimens were more likely to be 16S rRNA PCR/sequencing positive than tissue specimens in clinically infected patients. In addition, mean CRP and ESR levels were higher in those with positive than in those with negative 16S rRNA PCR/sequencing results ($P < .001$). This is in contrast to previous findings in which histopathologic findings of inflammation rather than systemic inflammation were related to the high rates of 16S rRNA PCR/sequencing positivity [28].

When blood and tissue cultures fail to identify a pathogen in culture-negative infective endocarditis, 16S rRNA PCR/sequencing may be a useful diagnostic tool [18]. High yield has been reported with 16S rRNA PCR/sequencing compared with bacterial cultures in blood culture-negative infective

endocarditis, with detection of fastidious organisms leading to a change in management in up to 15% of the cases [29]. In the current study, rates of 16S rRNA PCR/sequencing positivity were highest for cardiovascular specimens, whereas bacterial cultures in this group were less likely than other specimen sources to be positive. In 2 cases, difficult-to-culture organisms (*M. hominis* and *L. clevelandensis*) were detected which may explain these findings. Another reason may be the high frequency of prior antimicrobial therapy, given in 70% of the cases with cardiovascular infection (Table 3). The high yield of 16S rRNA PCR/sequencing from cardiac valves from patients with infective endocarditis may be related to persistence of bacterial DNA in valves for months to years even with successful treatment [30, 31]; 16S rRNA PCR/sequencing of valve tissue has been incorporated in the diagnostic algorithm of infective endocarditis given the high sensitivity and specificity with this specimen type [32].

Past studies have shown that molecular diagnostics may lead to changes in clinical management in infectious syndromes in up to 4%–15% of cases [27, 28]. Overall, in the current study, 16S rRNA PCR/sequencing led to a change in management in 5% of cases. This effect was proportionally higher in cases involving testing of cardiovascular specimens (22%), compared with other specimen types. Among MSK specimens, the overall PCR positivity rate was 30% in the clinically infected group. However, it led to change in management in only 6% of the cases. The previously reported yield of 16S rRNA PCR/sequencing in the diagnosis of prosthetic joint infection was 23%–32%, with an impact on clinical care in 15%–72% of cases [18, 20, 33]. Overall, positive 16S rRNA PCR/sequencing results affected clinical care more than negative results as patients with a high suspicion for underlying infection may receive treatment regardless of culture or 16S rRNA PCR/sequencing results. However, even in 16S rRNA PCR negative cases, antimicrobial therapy was either de-escalated or discontinued in 8 patients, highlighting the potential utility of such testing as an antimicrobial stewardship tool [34].

The overall sensitivity of bacterial cultures and 16S rRNA PCR/sequencing for diagnosis of infection was low. Previous studies have compared the sensitivity and specificity of 16S rRNA PCR/sequencing with culture as a reference standard; however, it is known that culture results are affected by prior antimicrobial therapy and may not detect fastidious organisms [35–38]. Studies comparing 16S rRNA PCR/sequencing to cultures have reported varying sensitivities and specificities, ranging from 43% to 96% and from 72% to 95%, respectively, depending on the tissue type and the patient population [3, 16, 39–41]. In the current study, we calculated sensitivity based on final clinical diagnosis, and with that criterion, the sensitivity of 16S rRNA PCR/sequencing and cultures were 30% and 34%, respectively, highlighting the limitation of culture-based tests as diagnostic tools and a concern with their use as a reference standard.

There are several limitations to 16S rRNA PCR/sequencing. Neither cultures (as typically performed clinically) nor 16S rRNA PCR/sequencing produce truly quantitative results (although 16S rRNA PCR/sequencing could be developed as a quantitative PCR-type assay by leveraging cycle thresholds and incorporating quantitative standards). Another limitation is that results do not provide information about antimicrobial susceptibility; 16S rRNA may detect nonviable organisms, which may or may not be clinically relevant. Moreover, as with microbiologic cultures, there is a risk of contamination. Depending on the sequencing technique used, 16S rRNA PCR/sequencing may (next-generation sequencing) or may not (Sanger sequencing, as used here) be able to detect multiple organisms in a sample. In addition, this technique is limited to bacterial pathogens and even in these cases, may not discriminate well between certain species (eg, *Mycobacterium* species) owing to high sequence similarities. The specific region of the 16S rRNA gene sequence could be changed to increase differentiation between related species.

Our study has several limitations. There may be selection bias influencing interpretation of results and generalizability; Mayo Clinic is a referral center, and the patient population was complex, with prolonged hospitalizations, multiple comorbid conditions, and antimicrobial exposure. In 22% of 16S rRNA PCR/sequencing positive cases, a specific organism could not be identified by sequencing. This is a limitation of Sanger sequencing that allows detection of only a single organism or 16S rRNA gene copy variant. This can be overcome by next-generation sequencing of the amplified 16S rRNA gene, but that approach can yield a complicated interpretive scenario and is more costly and generally slower than Sanger sequencing. Finally only samples with cycle threshold of <32 cycles underwent sequencing. Those with higher cycle threshold values (considered negative here) might yield clinically valuable results, especially with next generation sequencing.

This study suggests that 16S rRNA PCR/sequencing is clinically useful with an important role in diagnostic evaluation of patients with culture-negative infections, especially cardiovascular infections. Optimizing specimen selection based on clinical suspicion of infection, infectious syndrome and systemic inflammation may increase the likelihood of a positive result. These advantages and limitations should be considered on an individualized basis before performing 16S rRNA PCR/sequencing in clinical practice.

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 copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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