Infection and Drug Resistance Dovepress

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Exploring the Microbial Landscape of Bone and Joint Infections: An Analysis Using 16S rRNA Metagenome Sequencing

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Background: Bone and joint infections (BJIs) are challenging to diagnose. This study evaluated the utility of 16S rRNA gene sequencing in diagnosing BJIs, comparing it with conventional bacterial culture to explore microbial diversity in orthopedic infections.

Methods: Thirty patients with BJIs were enrolled from January 2019 to September 2020 at a single orthopedic center. Diagnoses were based on the Musculoskeletal Infection Society standards. DNA extraction, 16S rRNA sequencing, and microbial composition analysis were performed. Conventional bacterial culture results were compared with metagenomics detection, and associations with blood routine and biochemical test factors were analyzed.

Results: The study enrolled 30 patients with BJIs. Traditional bacterial culture successfully identified pathogens in 60% (18/30) of cases, predominantly *Staphylococcus aureus*. In contrast, 16S rRNA metagenomics sequencing revealed distinct microorganisms in all cases, it unveiled a diverse microbial landscape. The correlation between bacterial culture and metagenomics detection showcased both concordance and discrepancies. Consistency of detection between the two methods showed that metagenomics detection detected the same genus or species in 14 (87.5%) of the 16 samples identified as species by bacterial culture. In nearly half of the patients with negative cultures, pathogenic microorganisms were detected, highlighting the capability of 16S rRNA sequencing to identify microorganisms, even in samples with negative or unidentified culture results. Moreover, no significant correlation was observed between bacterial culture, metagenomics detection and the factors of blood routine and biochemical test.

Conclusion: This study deepens our understanding of the microbial complexity in BJIs. While traditional culture methods are cost-effective and practical, 16S rRNA gene sequencing proves valuable for complementary microbial analysis, particularly when traditional methods fail or rapid identification is critical. This emerging diagnostic approach can enhance the accuracy and speed of pathogen identification, enabling more effective interventions in the management of BJIs.

Keywords: bone and joint infections, 16S rRNA gene sequencing, microbial complexity, diagnostic tool, clinical management

Introduction

Bone and joint infections (BJIs) pose significant challenges in clinical management, often resulting in severe morbidity and long-term complications.[1,2] BJIs constitute a multifaceted spectrum of inflammatory conditions, encompassing osteomyelitis, septic arthritis (SA), prosthetic joint infections (PJI), spinal infections and diabetic foot osteomyelitis.[3] These infections can arise through hematogenous spread, contiguous extension from nearby tissues, or direct inoculation during surgical procedures or trauma.[4,5] The incidence of SA is estimated at 4 to 12 cases per 100,000 person-years.[6] In Germany, BJIs have an estimated incidence of approximately 24 per 100,000 for PJI, 17 per 100,000 for osteomyelitis, and 11 per 100,000 for FRI.[7] In a study conducted in Northeast China, the prevalence of FRI was around 1.5%.[8] Although a Canadian study showed a slight decrease in the risk of PJI over the past 15 years, PJI remains one of the most concerning complications.[9] In the United States and other regions worldwide, studies indicate that with the aging population, the incidence of BJIs is on the rise.[10,11] These infections not only significantly compromise the quality of life for affected individuals but also impose a substantial burden on healthcare systems.[12] The determination of the

Received: 7 August 2024
Accepted: 2 December 2024
Published: 13 December 2024

causative pathogen significantly influences the selection of optimal systemic antibiotic therapy. Hence, the prompt and precise identification of pathogens constitutes a pivotal phase in the successful management of BJIs.

BJIs can be caused by a variety of bacteria, and the most prevalent pathogens include *Staphylococcus aureus*, coagulase-negative staphylococci (CoNS), as well as other causative organisms such as *Escherichia coli, Enterobacteriaceae, Pseudomonas aeruginosa, Acinetobacter baumannii, Mycobacterium tuberculosis*, and others.[3,5] However, traditional diagnostic methods often face limitations in accurately identifying the causative agents, hindering timely and targeted therapeutic interventions. The gold standard, microbiologic laboratory culture, faces limitations in identifying bacteria within biofilms due to restrictive growth conditions.[13] Molecular biology advancements offer a hopeful prospect in addressing these challenges, paving the way for more precise diagnostic solutions.

In the recent decade, several new laboratory identification techniques have been used in the clinical pathogen detection, such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), genomic sequencing and implant sonication.[14,15] Among these approaches, molecular techniques for bacterial detection, particularly the use of 16S rRNA metagenomics has emerged as a powerful tool in unraveling the complexity of microbial communities in cancers and bacterial infections[16–20]. The 16S rRNA gene, a conserved region in bacterial genomes, allows for the identification of bacterial species by sequencing and analyzing this marker. This molecular technique enables a culture-independent, comprehensive analysis of the microbial composition, providing a more accurate representation of the diverse bacterial communities involved in these infections.[21] Additionally, the simplicity of data analysis compared to whole-genome sequencing methods enhances its accessibility, making it a practical choice for laboratories with varying levels of expertise and resources.

Recognized for its heightened specificity and discriminatory power in identifying bacterial species, 16S rRNA gene sequencing stands as a valuable tool in the diagnosis of bacterial infections. Despite the gradual acknowledgment of its advantages, the application of 16S rRNA gene sequencing in the context of BJIs remains a subject of controversy. This study endeavors to enrich our comprehension of the potential value of 16S rRNA gene sequencing in clinical diagnostic procedures for these particular infections.

Materials and Methods

Patients with Articular Infections Enrolled in This Study

Patients with patients with bone and joint infection were enrolled in a single center in Beijing, China for our study from January 2019 to September 2020. The infection was diagnosed based on the standard of Musculoskeletal Infection Society (MSIS) and FRI diagnostic criteria.[22,23] Commonly, the diagnosis of infection is based on a combination of clinical findings, laboratory results from peripheral blood test and synovial fluid, microbiological culture, histological tests, and intraoperative findings. Fluid samples submitted for testing were collected on admission of the patients. Direct plating was used for microorganism identification as part of the conventional bacterial culture methods. The clinical data including blood parameters for these patients were obtained by reviewing their medical records. The study was conducted in accordance with the Declaration of Helsinki and submitted to, and approved by, the local Ethics Committee of Beijing Luhe Hospital, Capital Medical University. Clinical samples and information were obtained after getting written informed consents from all participants.

Collection of Samples and DNA Extraction

For patients with infections located in the hip, knee, or shoulder, joint fluid was aspirated, while fluid aspirated or collected directly from the infected sites in other cases. Approximately 5–10 mL of joint fluid or other collected fluid samples were processed under sterile conditions and centrifuged at 4500g for 15 minutes at 4°C in a 50 mL tube. Then, the pellets were re-suspended in a total volume of 1 mL sterile phosphate-buffered saline (PBS) and transferred into a 1.5 mL sterile centrifugation tube and stored in −80 °C immediately. DNA was extracted using MagNA Pure LC 2.0 System and MagNA Pure LC Total NA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and quantified using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Oregon, USA). Distilled water

was processed using the same extraction protocol to serve as a negative control. During data analysis, sequencing results from the negative control were utilized to identify and exclude any potential contaminants detected.

16S rDNA High Throughput Sequencing

Polymerase Chain Reaction (PCR) amplification of V3-V4 region was performed with the following primers containing Illumina adapter sequences and dual-index barcodes used for tagging each sample: 341F 5'-CCTACGGGNGGCWGCAG-3' and 805R 5'-GACTACHVGGGTATCTAATCC-3'. The PCR reaction condition was as followed: 95°C for 3 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. PCR products were cleaned-up using AMPure XP Beads (Item No. A63882, Beckman Coulter Inc, CA, USA). The amplicon sequencing libraries were constructed in accordance with the 16S Metagenomic Sequencing Library Preparation (Illumina, Inc., San Diego, CA). Paired-end sequencing was performed on a Miseq platform to obtain 250-bp read (Illumina, Inc., CA, USA).

Analysis of the Microbial Composition

The sequencing reads were processed using Quantitative Insights into Microbial Ecology (QIIME, version 1.9.0), and an index of alpha diversity was calculated with QIIME based on sequence similarity at 97% OTU. Filtered sequences were clustered into Operational taxonomic units (OTUs), with 97% identity, and assigned to taxonomy using the Greengenes Database. The relationship of microbial composition of the patients was assessed by analysis of Bray-Curtis distance, these metrics assessed the phylogenetic similarity of bacterial community pairs, considering OTU relative abundance or presence/absence, respectively. Pearson correlation analysis was conducted to assess relationships between identified microbial profiles with blood tests. These correlations were analyzed to determine whether specific microbial communities were associated with distinct inflammatory responses.

Results

The Clinical Feature of Patients Enrolled in This Study

Thirty cases were enrolled in the study in total (Table 1). The mean age of the patients was 61.6±13.4 years and 14 (46.7%) were females. The patients had experienced surgeries mainly including total joint arthroplasty (12 patients), fracture internal fixation (14 patients). Sample sources were including 11 knees, 5 hips, 4 tibias, 3 spines and others. Most infections occurred in patients with joint implants or fracture internal fixation devices. These patients commonly presented with typical symptoms such as local redness, swelling, warmth, and pain, often accompanied by systemic symptoms like fever. In some cases, sinus tract formation was also observed. For spinal infections, patients primarily exhibited severe back pain and restricted movement. These clinical presentations provided important guidance for the initial diagnosis and sample collection. The laboratory tests showed that four patients had increased counts of white blood cells (WBC), 17 had increased level of C-reaction protein (CRP), and 21 had increased level of erythrocyte sedimentation rate (ESR). Based on these symptoms and results of tests, the patients were diagnosed as articular infection and the joint fluid samples were collected immediately (one sample each patient) before the treatments with antibiotics.

Bacterial Culture Results of Fluid Samples

By conventional bacterial culture, the samples from 18 patients were positive for at least one bacterial species (Table 1), accounting for a positive rate of 60% (18/30). The bacterial species were all common pathogens of clinical opportunistic infections, including *Staphylococcus aureus* (10 patients), *Staphylococcus epidermidis* (2 patients), *Staphylococcus haemolyticus* (1 patient), *Streptococcus agalactiae* (1 patient), *Escherichia coli* (1 patient), and *Pseudomonas aeruginosa* (1 patient). One patient was positive for both *S. epidermidis* and *P. aeruginosa*. In two patients, the culture results were positive for G+ coccus and G+ bacilli, respectively, however the taxonomy of the isolates cannot be identified.

Table 1 Clinical Characteristics of Patients Enrolled in This Study

No	ID	Age	Sex	Sample Source	Surgery*	Sampling Time Since Surgery	Culture Results
1	P01	80	M	Hip	THA	8 years	*Staphylococcus aureus*
2	P02	75	F	Knee	TKA	1 years	*G+ coccus*
3	P03	60	F	Knee	ORIF	1 week	*NA*
4	P04	28	F	Knee	ORIF	3 days	*Staphylococcus aureus*
5	P05	75	M	Tibia	ORIF	4 days	*Staphylococcus haemolyticus*
6	P06	61	M	Tibia	ORIF	7 days	*Staphylococcus aureus*
7	P07	47	F	Knee	TKA	2 years	*NA*
8	P08	51	M	Knee	TKA	10 months	*Staphylococcus aureus*
9	P09	67	M	Knee	TKA	10 years	*Staphylococcus epidermidis*
10	P10	66	F	Calcaneus	ORIF	5 days	*Staphylococcus aureus*
11	P11	71	F	Knee	arthroscope	4 days	*Staphylococcus aureus*
12	P12	33	M	Ankle	Achilles Tendon suture	5 days	*NA*
13	P13	59	M	Buttock	cystectomy	6 days	*Staphylococcus aureus*
14	P14	63	M	Spine[#]	ORIF	6 days	*Escherichia coli*
15	P15	82	F	Hip	ORIF	1 week	*Pseudomonas aeruginosa*
16	P16	64	M	Ankle	ORIF	29 days	*NA*
17	P17	65	F	Knee	TKA	6 years	*NA*
18	P18	58	M	Spine[#]	ORIF	5 days	*NA*
19	P19	68	M	Knee	TKA	3 months	*NA*
20	P20	69	F	Hip	THA	8 weeks	*NA*
21	P21	62	F	Knee	TKA	2 years	*NA*
22	P22	49	M	Tibia	ORIF	35 days	*Staphylococcus aureus*
23	P23	81	F	Shoulder	TSA	8 years	*Staphylococcus aureus*
24	P25	56	M	Spine[#]	ORIF	7 days	*NA*
25	P26	70	M	Hip	ORIF	3 weeks	*NA*
26	P27	35	M	Knee	MPFL Recon	7 days	*NA*
27	P28	53	M	Elbow	ORIF	5 days	*G+ bacilli*
28	P30	66	F	Tibia	ORIF	28 days	*Staphylococcus aureus*
29	P31	62	F	Hip	THA	19 years	*Streptococcus agalactiae*
30	P32	72	F	Hip	THA	5 years	*Staphylococcus epidermidis, Pseudomonas aeruginosa*

Notes: [#]Spine sample source include:Thoracic intervertebral, Lumbar Vertebra, cervical spine.
Abbreviations: *THA, Total hip arthroplasty; TKA, Total knee arthroplasty; TSA, Total shoulder arthroplasty; ORIF:,Open Reduction Internal Fixation; NA, Not Available.

The Bacterial Composition Detected in Fluid Samples by 16S rDNA Metagenomics

Using 16S rRNA metagenomic sequencing, we analyzed the microbiome of these samples and identified several typical pathogens, along with a variety of commensal and environmental bacteria (Figures 1 and 2). Notably, OTUs assigned to the genus *Staphylococcus* were detected in samples from 14 patients (14/30, 46.7%), accounting for 19.3% ± 15.9% of the microbiome. Among these, OTUs assigned to *S. aureus* were present in 13 samples, comprising 13.8% ± 10.5% of the microbiome (Figure 1). These findings suggest that *S. aureus* is a dominant pathogen causing BJIs in our hospital. Additionally, OTUs assigned to the genus *Streptococcus* were detected in samples from four patients, and *S. agalactiae* was identified in one patient (Figure 3).

We also detected OTUs assigned as *Haemophilus parainfluenzae* and *Prevotella melaninogenica*, each in one patient. In addition, presence of a complex synovial microbiome community has been detected besides these pathogenic bacteria, including the genera of *Sphingomonas, Comamonas, Burkholderia, Porphyromonas, Mesorhizobium, Agrobacterium, Finegoldia*, etc.

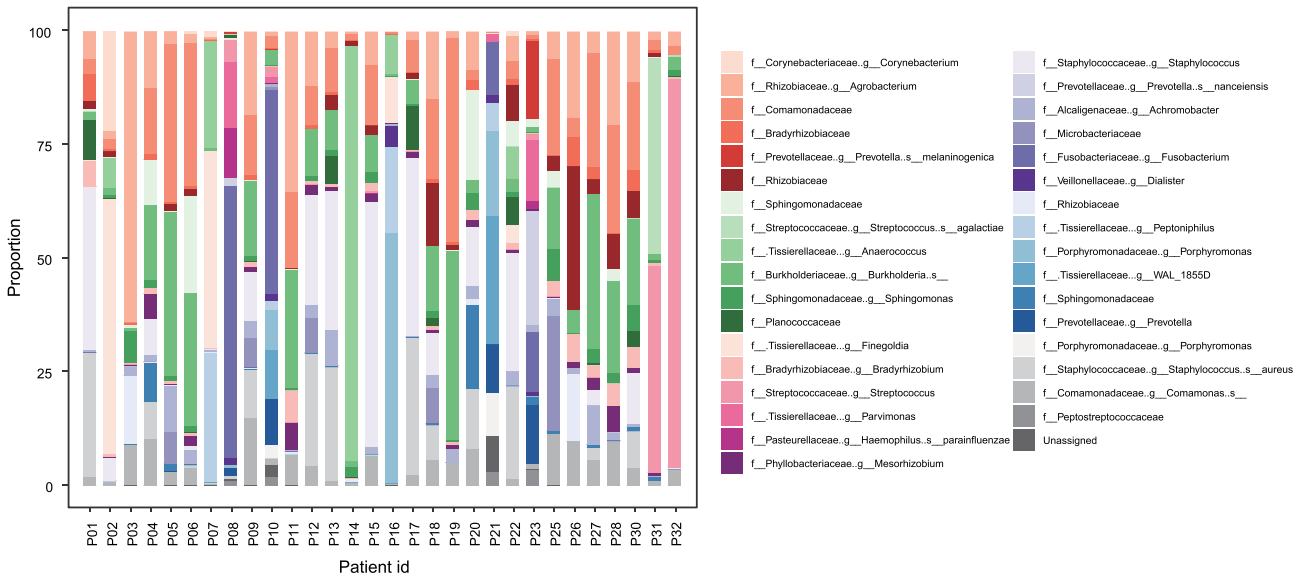

Figure 1 Bacterial composition of the 30 samples.

The Consistency of Bacterial Culture and Metagenomics Detection

By comparison of the bacterial culture and metagenomics detection results, we calculated the consistency of results from the two methods. Among the 16 samples with bacterial cultures identified at species level, we detected corresponding OTUs assigned as the same genus or species in 14 (87.5%) samples (Figure 3). In five samples the OTUs corresponding to the cultures were in the top three of each sample, and in two samples the OTUs corresponding to the cultures took the proportion of 7%~10%. However, in other 7 samples, proportions of the OTUs corresponding to the cultures were lower than 1%. In contrast, Among the 14 samples with negative (12) or unidentified (2) culture results, we also did not detect pathogenic bacteria in eight samples (8/14, 57.1%) by metagenomics, whereas in other six samples *Staphylococcus* were the dominant OUT (Figure 2, Supplementary Table 1). These might be caused by the low positive rate of conventional bacterial culture commonly in clinical laboratories.

The Correlation of Bacterial Culture, Metagenomics Detection and the Results of Blood Routine and Biochemical Test

We also calculated the correlation of bacterial culture, metagenomics detection and the factors of blood routine and biochemical test associated with infection (Supplementary Tables 1 and 2, Supplementary Figure 1). However, no significant correlation was observed. This may be attributed to the diversity of pathogens causing BJIs, the variability of infection sites, and host immune status differences, along with the fact that peripheral blood tests sometimes fail to provide a direct reflection of the local infection conditions.

Discussion

In current clinical practice, conventional laboratory culture remains the routine method for detecting pathogens in BJIs. Despite being effective in identifying causative microorganisms and facilitating successful treatment in many cases, traditional bacterial culture methods exhibit several drawbacks. Firstly, the traditional bacterial culture exhibits a relatively low positivity rate, and literatures reports that the negativity rate for culturing pathogens in BJIs is up to 40%, emphasizing the challenges in accurately diagnosing.[24–26] Although recent studies indicate higher positivity rates, reaching around 80%, particularly when utilizing sonication fluid from prosthetic devices, this improvement still underscores the persistent limitations of traditional culture techniques.[27] Furthermore, the time required for bacterial culture is prolonged, ranging from 6 to 14 days.[28,29] This extended timeframe can sometimes lead to delays in initiating early and targeted antibiotic therapy, potentially compromising optimal patient outcomes. These limitations necessitate the

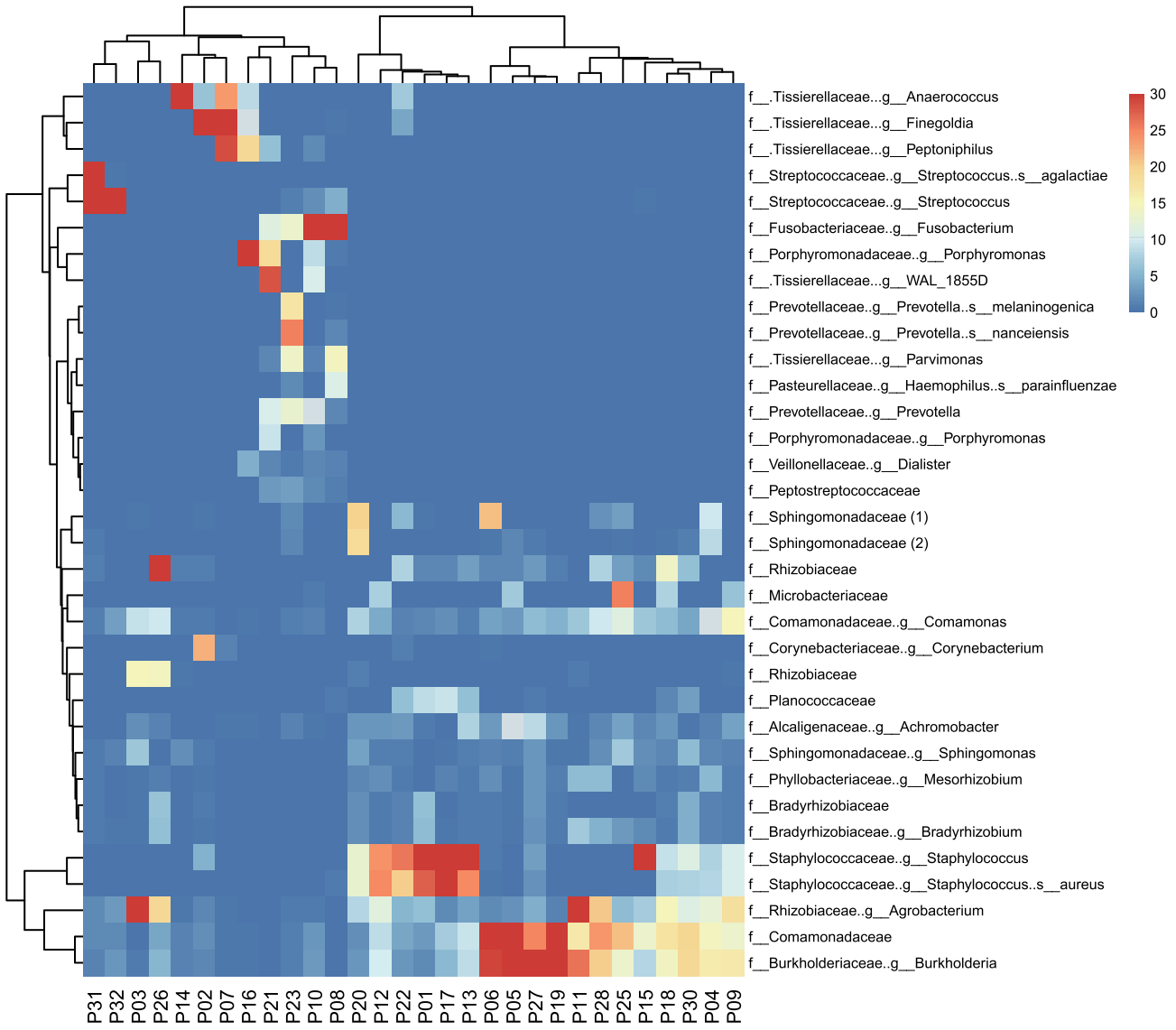

Figure 2 Cluster analysis based on the bacterial composition of the 30 samples.

exploration of alternative diagnostic approaches, such as molecular techniques including 16S rRNA gene sequencing, to overcome the challenges posed by traditional bacterial culture methods. Molecular methods offer the advantage of faster results and increased sensitivity, enabling a more timely and accurate identification of pathogens in musculoskeletal infections.[13,19,30]

Our study revealed a diverse microbial landscape in orthopedic-related infections, with *Staphylococcus aureus* being the most prevalent pathogen, aligning with established literature on common causative agents of BJIs.[31,32] The detection of multiple classic genera, including Streptococcus, Escherichia, and Pseudomonas, underscores the polymicrobial nature of these infections, emphasizing the importance of comprehensive diagnostic approaches. Moreover, the identification of microbes, such as *Haemophilus parainfluenzae* and *Prevotella melaninogenica*, highlights the complexity of microbial communities associated with BJIs. The presence of a broad range of bacterial genera indicates the need for thorough diagnostic methods capable of capturing the diversity inherent in these infections. A recent multicenter study by Goswami et al confirmed the presence of a microbiome in native knee and hip joints. Using 16S rRNA sequencing, microbial signals were detected in the joints of 113 patients, with the most abundant genera being *Escherichia, Cutibacterium, Staphylococcus, Acinetobacter*, and *Pseudomonas*.[33] These findings bear some similarities to our own

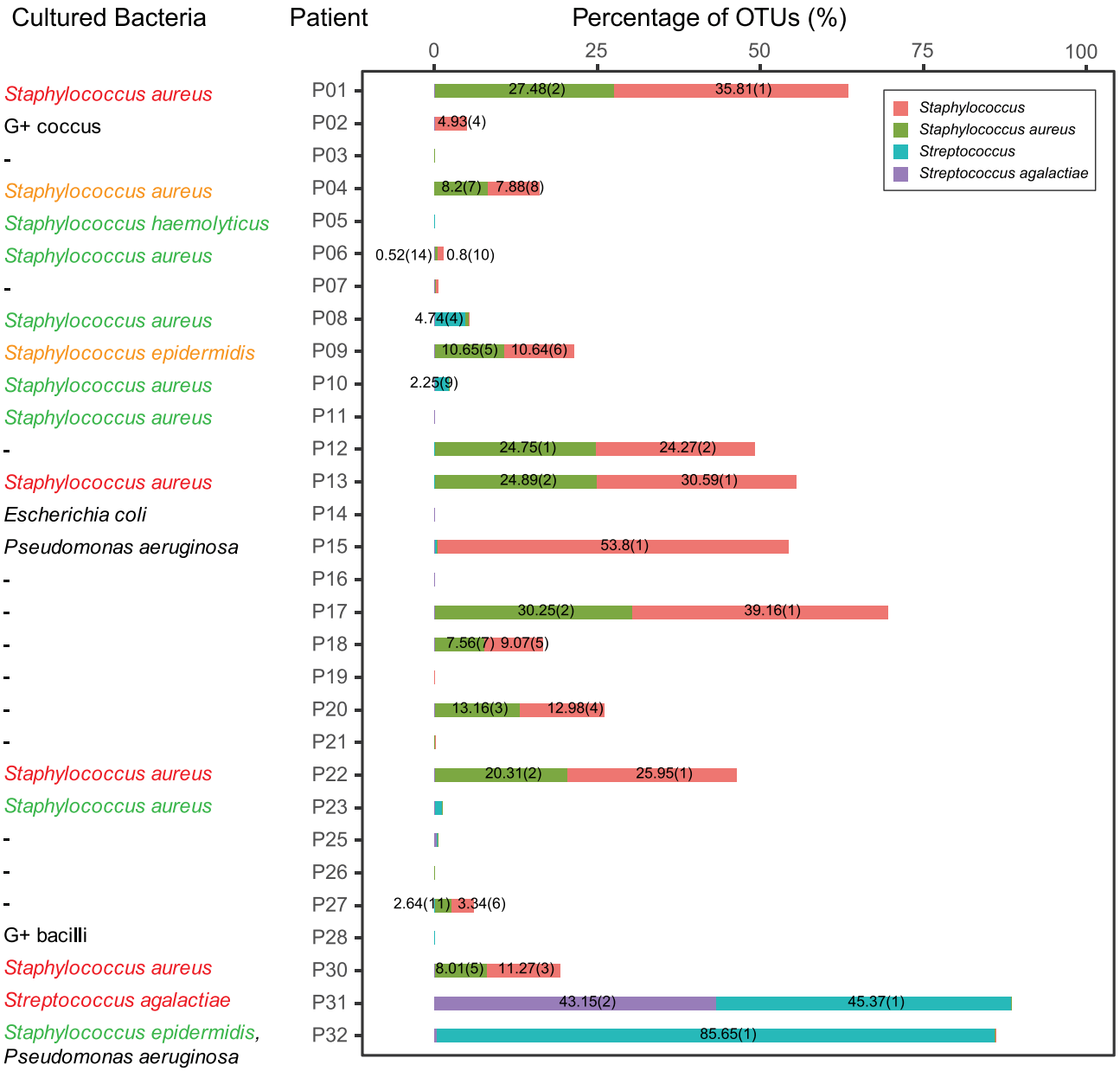

Figure 3 Consistency of bacterial culture and metagenomics detection. The bar plot at right shows the OTUs assigned as *Staphylococcus, Staphylococcus aureus, Streptococcus* and *Streptococcus agalactiae* which are also detected by culture. The proportions of OTUs are marked on the bar and the order of each OTU in the sample is in the bracket. The culture results are displayed at left. The species in red represent the corresponding OTUs are in top three of the sample, those in Orange represent the corresponding OTUs are dominant but not in the top three, and those in green represent the proportion of corresponding OTUs are less than 1%.

study results. Consistent with the findings reported by Hammad and Zhang et al, our study also supports the conclusion that a microbiome may be present in synovial fluid.[34,35]

In contrast to Natoli et al[13] who showed low concordance between second-generation DNA sequencing and bacterial cultures in orthopedic trauma surgery, our study showed higher concordance (87.5%) in detecting the corresponding OTUs at the genus or species level in samples that were positive for bacterial cultures, suggesting that 16S rRNA gene sequencing is reliable in identifying causative agents. The comparison between conventional bacterial culture and 16S rRNA metagenomic sequencing demonstrated both concordance and discrepancies. However, the study also noted instances where the abundance of corresponding OTUs was low, highlighting potential challenges in sensitivity.

16S rRNA metagenomic sequencing can detect microorganisms even when traditional cultures yield negative results.[36] In this study, microbial presence was identified in patients with negative traditional cultures, with nearly

40% of specimens showing the predominance of *Staphylococcus*, a pathogenic genus. In samples where traditional culture results were negative or unidentified, traditional cultivation methods might fail to identify certain microorganisms, especially those within bacterial biofilms. The presence of biofilms complicates the infection process and reduces the efficacy of antimicrobial agents.[37] Therefore, the 16S rRNA gene sequencing technique has important significance in detecting biofilm-forming bacteria. We would like to note that patients with early postoperative infections are often prone to culture-negative results due to prior antibiotic use. In such cases, 16S rRNA gene sequencing performs well as a complementary diagnostic tool, providing valuable insights that can support clinical decision-making despite culture limitations. And 16S rRNA gene sequencing has the capability to identify a broader range of pathogens, including those potentially missed by traditional cultures, indicating its potential as a supplementary or alternative diagnostic tool.[38]

Studies have reported that 16S rRNA metagenomic sequencing can detect some atypical pathogens in BJIs. Wilson et al,[39] utilizing next-generation sequencing (NGS), accurately diagnosed infections caused by uncommon pathogens in clinical cases where conventional test results were negative, allowing for timely intervention. The application of 16S rRNA metagenomic sequencing significantly reduces the time required for detection. Prompt identification of the pathogen through sequencing enables early intervention, contributing to proactive disease management. Chen et al noted a reduction in the diagnostic period from one week to two days, and the method can also detect pathogens with very low abundance, particularly in patients who have received antibiotic treatment before surgery.[17] The authors claim that 16S rRNA metagenomic sequencing is not only rapid and highly accurate but also capable of simultaneously detecting multiple pathogens, thereby avoiding potential misdiagnoses. Additionally, this study found no significant correlation between peripheral blood test indicators, bacterial cultures, and 16S rRNA sequencing results. This suggests that in practical clinical practice, when an infection is suspected but not indicated by peripheral blood or culture tests, molecular diagnostic methods should be promptly employed.

This study has certain limitations. While the sample size provides valuable insights, it may not fully represent the diversity of BJIs. Future larger-scale studies are needed to further validate and extend the diagnostic value within specific pathogens. Additionally, the lack of clear correlation between microbial detection and routine blood and biochemical tests highlights the localized nature of joint infections. Future research could explore other specific markers for joint infections to enhance diagnostic accuracy. Despite strict management and standardization of materials, reagents, and procedures, potential sample contamination remains a concern, although we utilized distilled water as a negative control and closely monitored the entire experimental process. Furthermore, it is important to note that 16S rRNA gene sequencing, while highly effective for the rapid identification of bacterial pathogens, lacks the capability to provide direct antibiotic susceptibility information. In BJIs, 16S rRNA metagenomic sequencing not only detects bacteria consistent with bacterial cultures but also identifies bacteria not detected by culture, providing faster and more accurate microbiological evidence for rapid clinical diagnosis—a valuable supplement to existing diagnostic methods.

Conclusion

In conclusion, this study enhances our understanding of the microbial complexity in BJIs. While traditional bacterial cultures remain crucial for detecting pathogens and determining antibiotic susceptibility in orthopedic-related infections, 16S rRNA gene sequencing adds significant value by providing a comprehensive, culture-independent analysis of microbial communities. As a supplementary diagnostic tool, 16S rRNA sequencing can improve the accuracy and timeliness of pathogen identification, thereby supporting more effective therapeutic interventions alongside standard culture-based methods in the challenging clinical management of BJIs.

Ethical Approval

This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 helsinki Declaration and its later amendments or comparable ethical standards. The Human Investigation Committee (IRB) of our hospital approved this study.

Acknowledgments

We would like to express our sincere gratitude to the study participants.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

Funded by the Youth research project LHYY2024-JC04, Beijing Luhe Hospital, Capital Medical University.

Disclosure

The authors declare that they have no competing interests.

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