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Original Article

Evaluation of infections in orthopedic patients using next-generation sequencing

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ABSTRACT

Introduction: Culture tests are used to diagnose infections, but there are various problems such as low sensitivity in detecting infections in orthopedic cases. To address this problem, next generation sequencing (NGS) analysis, which can comprehensively search for bacterial genes, is being applied clinically. In this study, we examined whether NGS analysis was useful in evaluating infections in orthopedic cases.

Methods: The participants were 23 patients suspected of having an infection between 2016 and 2017. Samples were collected from tissues suspected of being infected and were subjected to culture tests and NGS analysis, and the positive rates from the culture tests and from the NGS analysis were compared. We also attempted to determine cutoff value for the NGS analysis.

Results: A total of 20 cases were ultimately diagnosed as infections and 3 cases were diagnosed as non-infections. The sensitivity of the culture tests was 70%, and the sensitivity of the NGS analysis was 55%. When the NGS analysis was performed with the diversity index set to the cut-off value, the sensitivity was 75% for the Simpson index. In this study, the sensitivity was 90% when the analysis was performed using the NGS index, which is a combination of the diversity index and the OTUs (operational taxonomic units) value.

Conclusion: NGS analysis using the NGS index showed excellent sensitivity and specificity compared to culture tests. NGS analysis is therefore a useful modality for assessing infections in orthopedic cases.

1. Introduction

In recent years, society has been aging due to advances in medicine, and the number of immunocompromised hosts has increased [1,2]. As a result, there has been an increase in cases of infection in the field of orthopedics. Although surgery might be indicated depending on the condition, antibiotic administration is still the gold standard for treating these infections.

To select an appropriate antibiotic, physicians need to identify the causative organism and conduct drug susceptibility tests. However, there are certain problems with using a conventional culture test for musculoskeletal infections, such as low sensitivity and false negative results [3,4]. In particular, the sensitivity of culture tests is low in cases in which antibiotics have been previously been administered [5]. Various methods have been proposed to solve these problems, such as blood culture bottles, ultrasonic preparation, infection markers, and polymerase chain reaction (PCR) tests [6–10]. However, culture tests cannot detect certain strains, and PCR tests cannot evaluate multiple or unknown organisms [11].

Next-generation sequencing (NGS) can detect all bacterial genes present in a sample [12]. By sequencing the 16s ribosomal RNA (rRNA) gene, the composition ratio of the microorganisms in the sample can be analyzed. The greatest feature of NGS analysis is that it can comprehensively detect all bacterial genes. On the other hand, one of the disadvantage of NGS analysis is that the sensitivity is too high and can be affected by contamination [13,14]. With NGS analysis, certain types of bacterial species are detected even in samples from patients without infections due to the influence of the normal flora of various organs or to contamination during sample processing. To overcome this issue, an indicator to distinguish true infections from non-infections is urgently

Abbreviations: AUC, Area under the curve; CRP, C-reactive protein; NGS, Next generation sequencing; OTU, Operational taxonomic units; PCoA, Principal coordinate analysis; PCR, Polymerase chain reaction; ROC, Receiver operating characteristic; WBC, White blood cell.

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needed. To the best of our knowledge, the study by Tarabichi et al. [15] is the only one on orthopedic infections to address this issue. The authors reported that a high percentage of specific bacterial genes could be detected in samples from infected patients, and the NGS detection of a single organism representing more than 59.5% of bacteria was a valuable criterion by which to distinguish patients with and without infections. With the exception of that study, methods to distinguish cases of infection from non-infection using NGS have been scarcely studied.

The present study aimed to investigate whether NGS analysis could overcome the disadvantages of culture tests and to explore a useful cutoff value with NGS for detecting infections in orthopedic cases.

2. Methods

2.1. Preoperative assessment

From 2016 to 2017, the study enrolled patients with possible infections based on interviews and physical findings. The target infections were pyogenic arthritis, pyogenic spondylitis, periprosthetic joint infection, osteomyelitis, postoperative wound infection, iliopsoas abscess, epidural abscess, and cellulitis. The final diagnosis of infection was achieved by multiple orthopedic surgeons who comprehensively evaluated the physical findings, blood tests, and culture tests. Blood tests assessed the white blood cell (WBC) count and C-reactive protein (CRP) level as inflammatory markers. We ultimately divided the patients into groups with infection or without infection, and we statistically compared age, sex, serum CRP, WBC, presence of purulent exudate, and pre-administration of antibiotics between the two groups. In all cases, samples were collected by surgery or biopsy and were submitted to a culture test. Culture tests and NGS analyses were performed on the same sample. The research protocol was approved by the institutional ethics board of Tottori university hospital. All participants provided written informed consent for the clinical and genetic studies.

2.2. Sample collection

Examination samples were collected during surgery or tissue biopsy and were subjected to a culture test first. The exudate from the lesion was collected with a sterile syringe. The collected specimens were transferred immediately to a clean Petri dish sent to the bacteria examination room. The remainder of the specimen sample was used for the NGS analysis.

2.3. Next-generation sequencing

Total DNA was extracted from 20 to 50 mg of tissue harvested during surgery using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. We assessed the purity of the DNA samples with a NanoDrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For all samples, ethanol precipitation was performed, resulting in a DNA concentration of 30 ng/μL. The purified DNA solution was diluted in a volume of 30 μL with distilled water and sent to Riken Genesis Co. Ltd (Kawasaki, Kanagawa, Japan).

The samples were subjected to a quality assessment, and DNA sequence analysis was performed using MiSeq sequencer (Illumina Co. Ltd., San Diego, CA, USA). The rRNA gene region (V3–V4) was amplified from the genomic DNA as specimen pretreatment. Next, a second PCR test was performed to add the sequences required at both ends for the sequence analysis.

The detected gene sequence was analyzed with the CL Community™ Version 3.30 (Chunlab Co. Ltd., Seoul, Republic of Korea) bacterial gene database and the strains were identified. The length of the library was 500–700 base pairs. The sequence analysis was performed using a flow cell as specified by MiSeq, and the read length was 251 base pairs by the multiplex method. We also added a process to remove short base sequences that could adversely affect the analysis. The sequence similarity of each nucleotide sequence was determined as a whole, and each cluster composed of a sequence having a similarity of greater than 97% was recognized as one bacterial species. We determined whether or not the nucleotide sequence of the bacteria detected by NGS was due to infection or contamination by setting a threshold, which was set based on the report by Tarabichi et al. [15]. The bacterial species accounting for 59.5% or more of all detected bacterial genes was considered to indicate infection. In addition, we also assessed a method using a diversity index as another threshold. Diversity indexes reflect the richness of and evenness in the sample and are employed to assess a sample's biological diversity. We calculated the operational taxonomic units (OTUs) and diversity indexes using CL Community™ software. The Shannon index is a positive value, with higher values indicating greater diversity (http://www.mothur.org/wiki/Shannon). The Simpson index ranges from 0 to 1, with 1 indicating the simplest microbiome composition (http://www.mothur.org/wiki/Simpson) [16]. We also performed three-dimensional projections based on the UniFrac distance matrix and principal coordinate analysis (PCoA) using this software.

2.4. Statistical analyses

All data are expressed as mean ± standard deviation. We employed Student's t-test to compare the differences in the continuous variables between two groups, such as age, serum CRP and serum WBC. We employed Fisher's exact test to compare the differences in the categorical variables such as sex, pus leakage, and prior antibiotic administration. We performed Welch's *t*-test assuming an unequal sample distribution variance to compare OTUs and indexes between the samples with and without infection. We performed receiver operating characteristic (ROC) analyses to estimate the overall diagnostic accuracy and defined the optimal cutoff value by the Youden index. We performed comparisons between the culture tests and NGS analyses using McNemar's chi-squared test. The statistical analysis was performed using mainly IBM SPSS Statistics version 25 (IBM, Tokyo, Japan). The results were considered significant when $p < 0.05$.

3. Results

This study enrolled 23 cases, 20 of which were ultimately diagnosed as infected, and 3 of which were diagnosed as uninfected. In the comparison between the patients with and without infection, those without infection were younger (Table 1).

The conventional culture test was positive in 14 cases (70%) in the infected group and 0 cases in the uninfected group. In the NGS analysis, enormous numbers of bacterial genes were detected in all cases. We therefore employed a diagnostic criterion proposed by Tarabichi et al.

Table 1
Demographic characteristic data for infection and non-infection patients.

Characteristic date	Infection (n = 20)	Non-infection (n = 3)	P value
Age	65.3 ± 4.6	28.3 ± 9.4	<0.01[a]
Sex			0.4[b]
male	8	2	
female	12	1	
Serum CRP (mg/dL)	9.14 ± 2.58 (0.03–36.38)	11.38 ± 11.27 (0.02–33.92)	0.36[c]
Serum WBC	9874 ± 1263 (3800–27980)	11933 ± 3815 (7300–19500)	0.55[c]
Leakage of pus	10	0	0.16[b]
Pre-antibiotics administration	6	1	0.79[b]

*WBC: White blood cell.
*CRP: C-reactive protein.
[a] Students t-test.
[b] Chi square test.
[c] Mann-Whitneys *U* test.

that considered a pathogen infectious when its genes accounted for 59.5% or more of all detected genes [15]. Based on this criterion, 8 (57.1%) of the culture tests positive for infection and 2 (33.3%) of the culture tests negative for infection were NGS positive, whereas no case was NGS positive in the uninfected group (Fig. 1). Therefore, the positive, negative, and total concordance was 57.1%, 77.8%, and 65.2%, respectively, indicating moderate concordance between the two types of tests (Table 2). Next, we examined the ability of the two tests to detect infection. The conventional culture test showed higher detection sensitivity (70%) than NGS (55%) using Tarabichi's criterion, although they had equal specificity (Fig. 2). To increase the ability of NGS to detect infection, we next sought a novel criterion for infection as determined by NGS. Tarabichi et al. demonstrated that culture-positive groups tended to detect higher percentages of a few bacterial genes, whereas culture-negative groups tended to detect lower percentages of a variety of bacterial genes [15]. In our data, the taxonomic composition chart of each sample showed the same tendency (Fig. 3). We then compared the species richness and evenness of the sample of patients with and without infection using OTUs and diversity indexes (the Simpson and Shannon indexes). As shown in Fig. 4A, the number of OTUs and the values of the Simpson index of the patients with infection were significantly higher than those of the patients without infection. We then examined the microbiome between those with and without infection using β-diversity calculated for the UniFrac distance. As shown in Fig. 4B, the microbial

Table 2
Comparison of NGS and culture test results.

NGS	Culture		Total
	Positive	Negative	
Positive	8	2	10
Negative	6	7	13
Total	14	9	23

Positive concordance 57.1% 8/14.
Negative concordance 77.8% 7/9.
Total concordance.
65.2% 15/23.
*NGS:Next generation sequencing.

structure of the microbiome of patients with infection markedly differed from that of those without infection. These data suggest that the samples from the patients with infection had greater species richness and lower species diversity than those without infection. Therefore, to explore the optimal cutoff value to distinguish infection from non-infection using NGS, we developed a novel index (the NGS index) calculated by multiplying the OTUs by the value of the Simpson index.

To estimate the indexes and cutoff values to distinguish patients with and without infection, we next performed an ROC analysis. In this analysis, we tested the Simpson index, Shannon index, and our newly developed NGS index. As shown in Fig. 5, the area under the curve

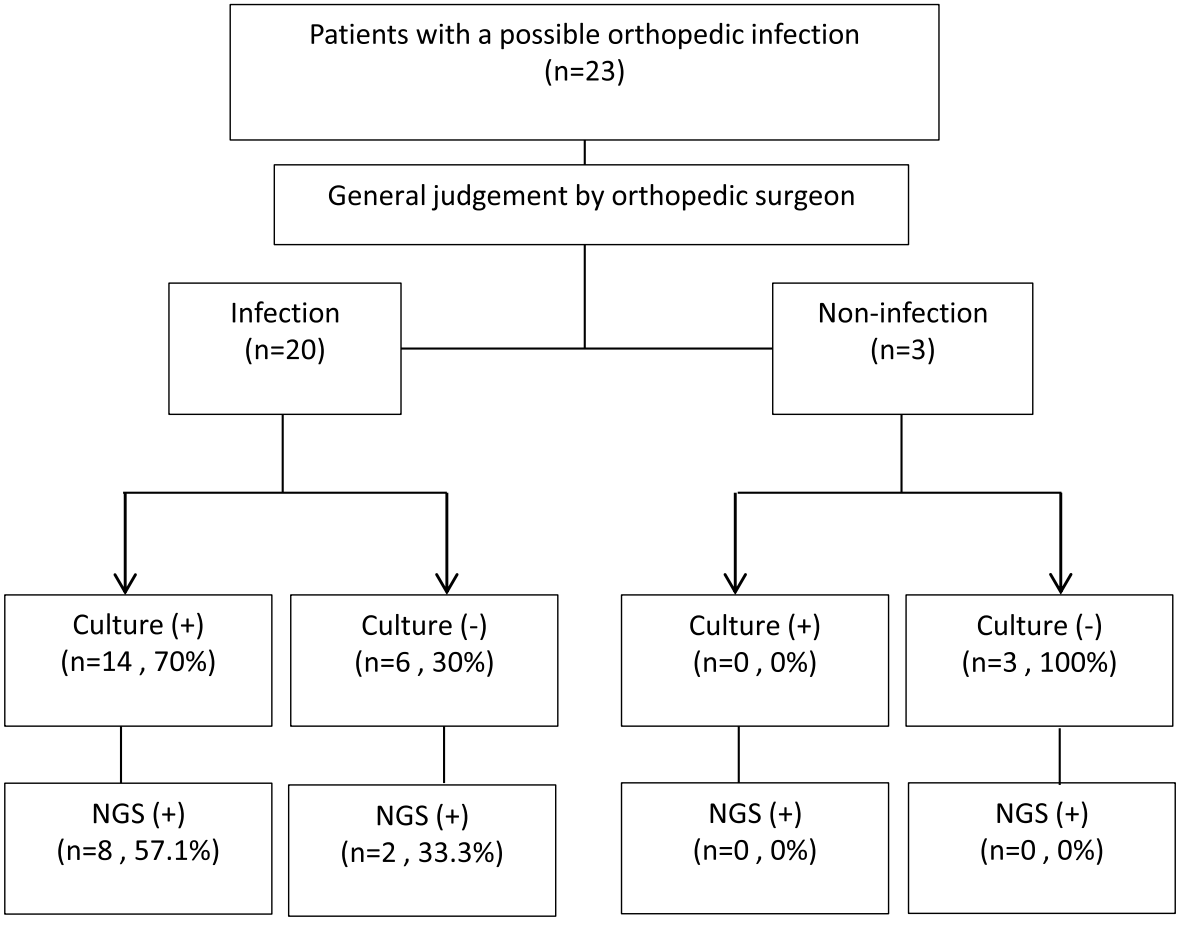

Fig. 1. Grouping of patients and results of bacteriological examinations.
A. Flowchart of the grouping of patients: In 20 patients with infections, 14 cases were culture test-positive. Among them, 8 cases (57.1 %) were also positive in the NGS analysis. In the patients without infections, neither the culture test nor the NGS analysis detected bacteria.
B. Concordance between culture tests and NGS analyses:
NGS: Next-generation sequencing
Culture (+): culture positive, Culture (−): culture negative, NGS (+): next-generation sequencing positive.

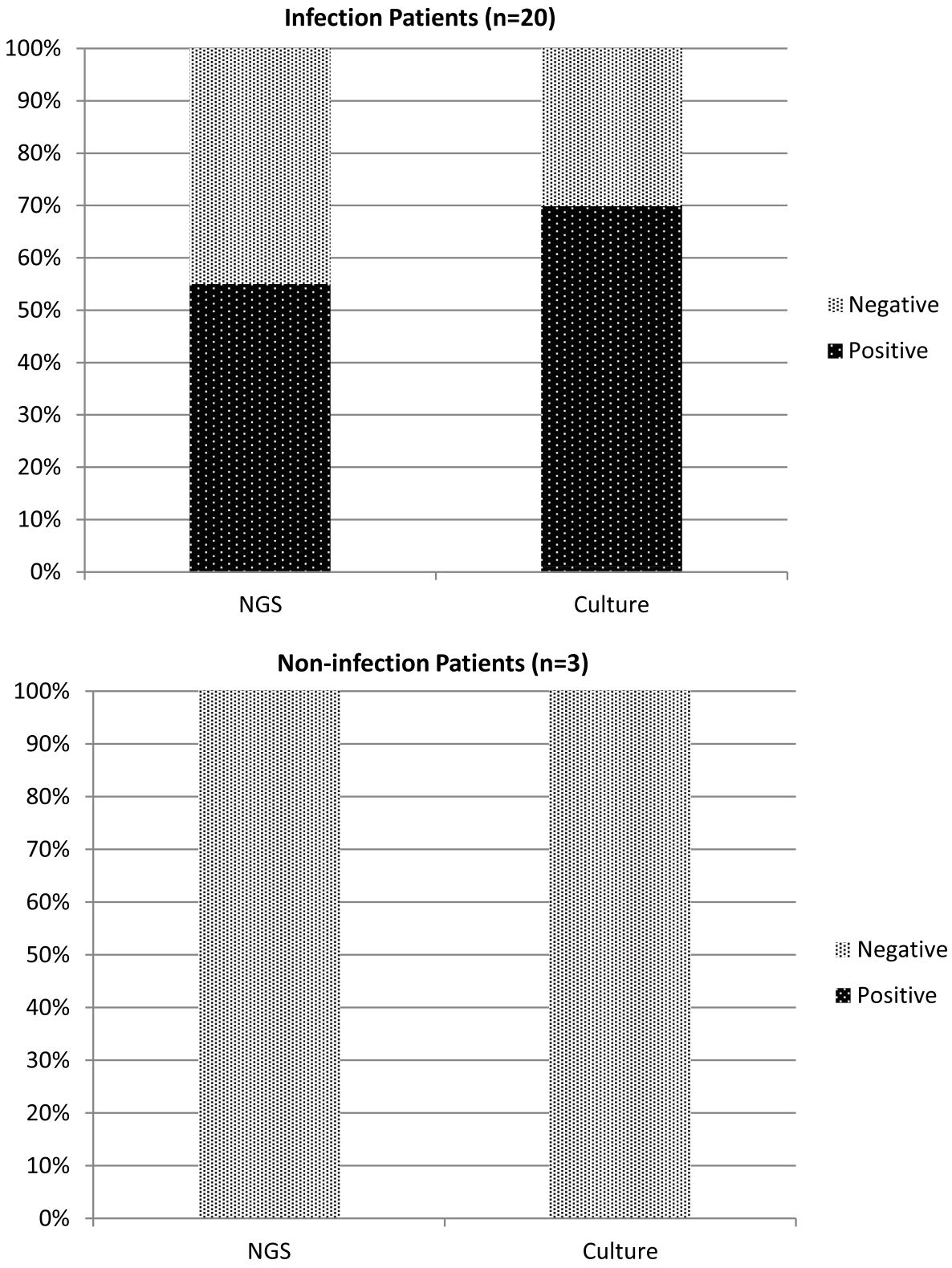

Fig. 2. Bar graph showing the comparison of NGS analyses and culture test results for patients with and without infections. The culture tests tended to be more sensitive than the NGS analysis, but both were similar in specificity.
NGS, Next-generation sequencing.

(AUC) for the Simpson index, Shannon index, and NGS index was 0.75, 0.67, and 0.93, respectively. The optimal cutoff value for the Simpson index, Shannon index, and NGS index was 0.064, 3.04, and 6 using a Youden index analysis, respectively. Therefore, the NGS index with a cutoff value of 6 was the best indicator to differentiate patients with and without infection using NGS.

We compared the sensitivity and specificity of NGS when applying Tarabichi's criteria, the Simpson index, and our NGS index with culture tests, the results of which are shown in Table 3. The analysis with our NGS index showed both the highest sensitivity (90%) and the highest

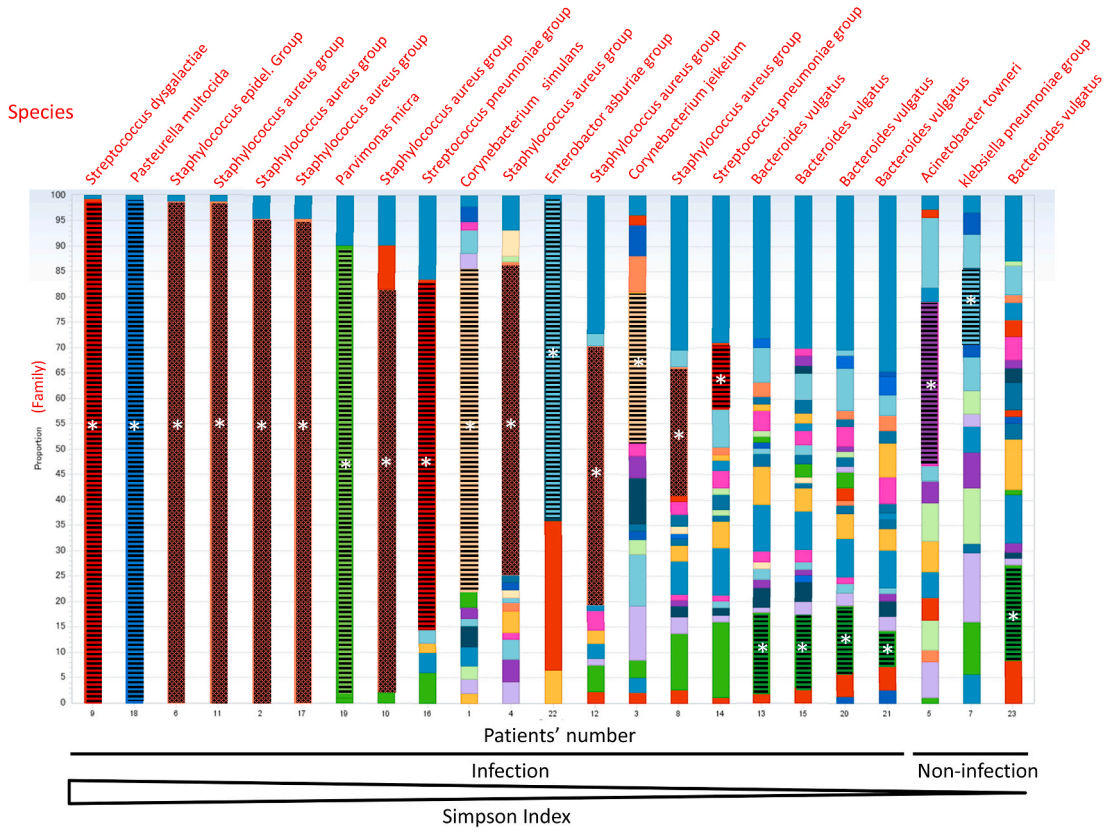

Fig. 3. Taxonomic composition (Family) of the samples from patients with and without infections.
The patient numbers were arranged in order of Simpson index. The microbiome in the group with infections tended to show low diversity. (In the Simpson index, the greater the value, the lower the diversity). Bacteria species listed above the graph are the most detected gene. The asterisk indicates the family to which the most content of bacteria belongs.

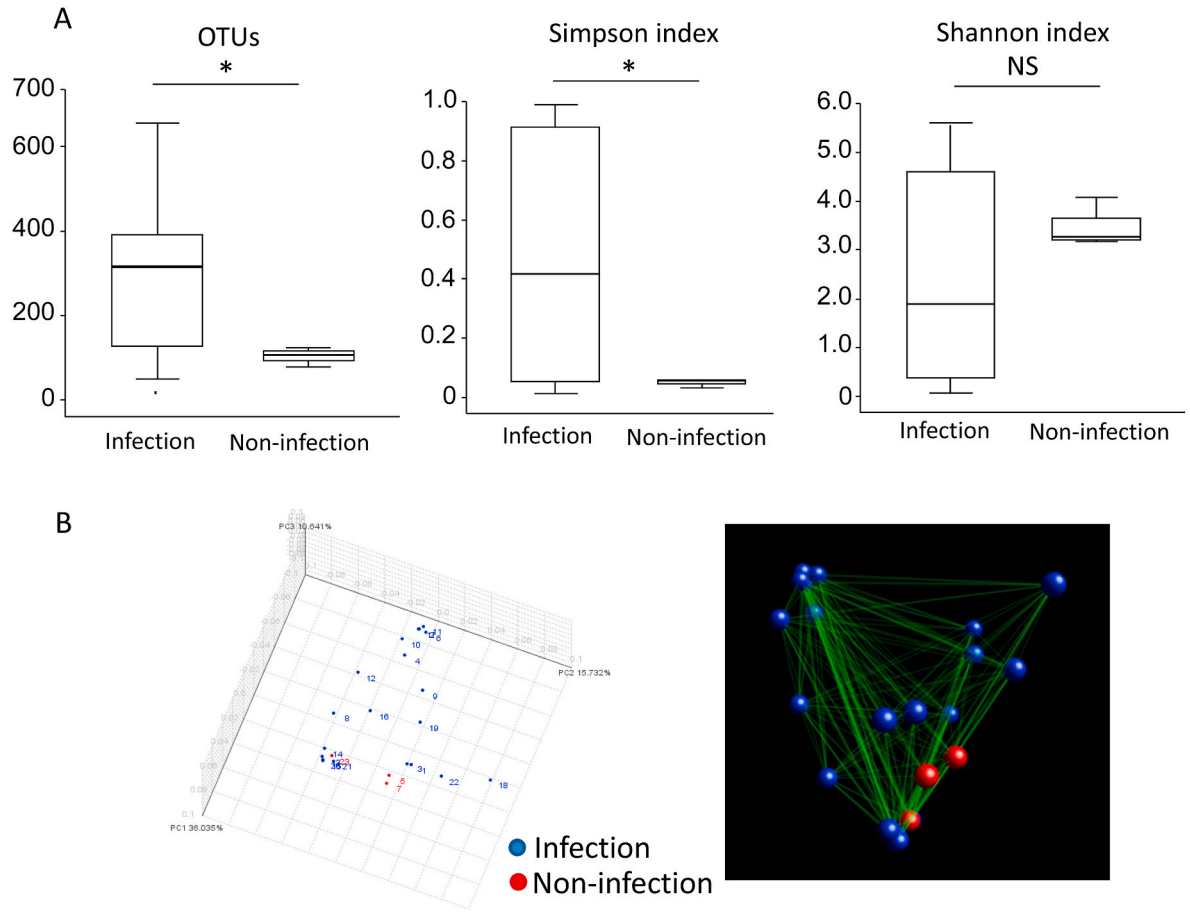

Fig. 4. Comparative analysis of the microbial community in the patients with and without infections.
A. The microbial community was compared using the indicators of species richness and evenness in the samples. We employed the number of OTUs and α-diversity indexes (Simpson and Shannon index) for this comparison.
OTUs, operational taxonomic units,
*p <
0.05 by
Welch's *t*-test assuming unequal sample distribution variance.
B. Comparison of the microbiome of the samples between patients with and without infections by PCoA of β-diversity measures. The microbial community was significantly different when comparing patients with and without infections.

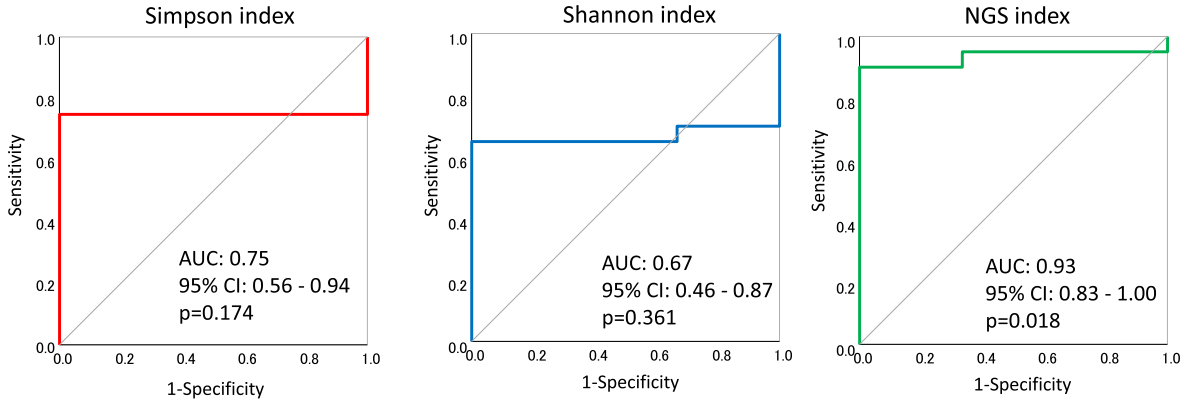

Fig. 5. ROC analysis using the diversity index (Simpson and Shannon index) and novel NGS index.
A significant AUC value (0.93) was obtained using the NGS index. The NGS index was the most useful for distinguishing patients with and without infection.
AUC, area under the curve; ROC, receiver operating characteristic.

specificity (100%), and these values were higher than those of the culture tests.

We compared the bacterial species detected by NGS and culture tests, the results of which are shown in Table 4. Among the infected patients, we observed NGS-positive and culture test-negative results in the samples with *Streptococcus* species (*Streptococcus pneumoniae, Streptococcus*

Table 3
Comparison of Simpson index, NGS index and culture test.

	Infection (n = 20)	Non-infection (n = 3)	Sensitivity	Specificity
NGS (59.5%< bacterial gene)			55%	100%
Positive	11	0		
Negative	9	3		
NGS (using Simpson index)			75%	100%
Positive	15	0		
Negative	5	3		
NGS (using NGS index)			90%	100%
Positive	18	0		
Negative	2	3		
Culture			70%	100%
Positive	14	0		
Negative	6	3		

*NGS:Next generation sequencing.

dysgalactiae) and anaerobes (*Bacteroides vulgatus*), which were fastidious bacteria or those that require anerobic conditions in culture.

4. Discussion

In this study, we compared the usefulness of NGS analyses against culture tests to distinguish orthopedic patients with and without infection. The NGS results indicated that the samples from the infected patients had greater species richness and lower diversity than those of the uninfected patients. We found that an NGS index calculated by multiplying the number of OTUs by the Simpson index provides the NGS analysis with higher sensitivity and specificity than culture tests in detecting infection. These data suggest that the combination of NGS and our novel NGS index is a potentially useful diagnostic modality for detecting infection in orthopedic patients.

The current study demonstrated 3 interesting findings. First, the NGS analysis detected bacterial genes even in culture test-negative patients. According to the reports on culture examinations for orthopedic infections, the positive rate was around 57%–70%; therefore, infection cannot be completely diagnosed by culture tests [3,4,17]. Of the 6 cases in this study that were negative for the culture test but were clinically diagnosed as an infection, 4 (67%) were positive according to the NGS analysis. In addition, NGS accurately diagnosed uninfected patients

Table 4
Comparison of causative organisms detected by NGS analysis and culture test.

NGS index	Patient	NGS	Culture	Infection	Prior antimicrobial therapy	Duration of administration (day)
437.895	19	Parvimonas micra	Parvimonas micra	+	-	
276.9392	17	Staphylococcus aureus group	MSSA	+	-	
252.8809	10	Staphylococcus aureus group	MSSA	+	-	
138.6469	11	Staphylococcus aureus group	MSSA	+	VCM+PIPC	3
138.3286	4	Staphylococcus aureus group	MRSA	+	CAM	9
133.5759	12	Staphylococcus aureus group	MSSA	+	-	
130.8648	16	Streptococcus pneumoniae group	negative	+	CEZ+CTRX	10
126.9512	18	Pasteurella multocida	Pasteurella multocida	+	MEPM	29
125.4156	9	Streptococcus dysgalactiae	negative	+	-	
89.06898	22	Enterobactor asburiae group	Enterobacter cloacae complex + Enterococcus faecalis	+	CCL	3
57.62904	6	Staphylococcus epidel. Group	Staphylococcus epidel.	+	-	
52.21309	2	Staphylococcus aureus group	MRSA	+	-	
27.93741	8	Staphylococcus aureus group	MRSA	+	-	
21.30762	1	Corynebacterium simulans	Corynebacterium striatum	+	-	
9.750509	14	Streptococcus pneumoniae group	Streptococcus mitis	+	-	
6.545602	21	Bacteroides vulgatus	negative	+	CEZ+CTRX	6
6.52767	13	Bacteroides vulgatus	negative	+	-	
6.142932	3	Corynebacterium jeikeium	MRSA	+	-	
5.858005	7	klebsiella pneumoniae group	negative	-	MPEM	2
5.304105	15	Bacteroides vulgatus	negative	+	-	
4.333154	5	Acinetobacter towneri	negative	-	-	
3.853616	23	Bacteroides vulgatus	negative	-	-	
3.791142	20	Bacteroides vulgatus	negative	+	-	

*Dotted line indicates the cutoff value of NGS index (6.0).
*CAM: Clarithromycin
*CCL: Cefaclor
*CEZ: Cefazolin
*CTRX: Ceftriaxone
*MPEM: Meropenem
*MRSA: Methicillin-resistant Staphylococcus aureus
*MSSA: Methicillin-sensitive Staphylococcus aureus
*NGS: Next generation sequencing
*PIPC: Piperacillin
*VCM: Vancomycin

(specificity, 100%). NGS analysis is therefore a promising and highly sensitive method for diagnosing infection in orthopedic patients.

Second, our results showed that in the cases diagnosed as infected (by NGS and not by the culture test), *Streptococcus* species and anaerobic bacteria were detected as infectious agents. These two bacteria shared the characteristics of being difficult to culture and needing special culture conditions. *Streptococcus* species are fastidious bacteria and need carbon dioxide conditions during culture, while anaerobic bacteria require anaerobic conditions. Our data suggest that using NGS for the diagnosis of orthopedic infections might improve the care for patients infected with these bacteria. For example, one of the patients included in this study was treated with antibiotics by a previous physician for fever and low back pain. After the patient's condition deteriorated, they were transferred to our hospital. After being admitted and after all the culture tests conducted at our hospital were negative, the patient's NGS analysis identified *Streptococcus* genes, and treatment was successfully administered. Given that this patient was previously administered antibiotics, an accurate diagnosis by culture test was difficult to achieve. Our experience demonstrates that NGS could overcome this problem. The use of NGS might improve our knowledge of the causative agents of orthopedic infection and improve the treatment for these patients.

The third finding is that the introduction of our novel NGS index improves the ability of NGS to distinguish infection from non-infection. Using NGS, a number of bacterial species are listed as a detected bacterium, even in the samples from patients without infection, due to bacterial contamination during the process of sampling or analytical procedure or the effect of pre-existing normal flora of the human body. It is therefore crucial to find a method to distinguish clinically relevant results from meaningless results when using NGS. To date, however, few studies have explored this issue. Tarabichi et al. observed that if more than 59.5% of bacteria belonging to a single species are found in NGS, the sample can be interpreted as NGS positive for orthopedic infection [15]. Liu et al. reported that the beta diversity of NGS analyses for oral microbiota can distinguish inflammation from non-inflammation after surgery for patients with cleft lips and palates [18]. Our study found that an increased number of OTUs and decreased diversity (as reflected by a higher Simpson index value) is associated with clinical infection. Based on these results, we devised a new NGS index that comprises OTUs and the Simpson index (the NGS index = OTU × Simpson index). We have shown that the NGS index has greater sensitivity than culture tests, while maintaining a high specificity comparable to culture tests. The combination of NGS and the NGS index is therefore a promising methodology to improve the care of patients with orthopedic infections.

There are certain limitations to this study. First, the number of cases was relatively small, and there were a variety of infections in the infected group. Second, the diagnosis of infection was clinically defined, given that there are no unified criteria for the broad range of orthopedic infections. To compensate for this limitation, the clinical diagnosis of infection was reached by multiple orthopedic surgeons without knowing the NGS results. Third, our NGS index cannot distinguish a mixed infection. In this study, the most detected bacterial gene using the NGS analysis was determined to be the causative organism, but it is unclear whether the second most detected bacterial gene was involved in the pathology. NGS analysis cannot rule out the possibility of mixed infections because it detects many bacterial genes. Therefore, NGS analysis is only an adjunct diagnosis. Fourth, NGS requires multiple steps and is time consuming compared with culture tests. However, improvements in sample processing and innovations in NGS methodology, such as the MinION system, will ultimately solve this problem. Fifth, there is no validation data using clear infectious samples when calculating the NGS index. Therefore, this study is an exploratory research about the NGS index. In order to apply the NGS index clinically, it is necessary to confirm the usefulness using verification data.

In conclusion, NGS analysis might identify causative organisms that are not detected by culture tests. The NGS index could be useful for identifying infections in orthopedic patients by employing NGS analysis.

Icmje statement

Contributors Shinya Ogawa was responsible for the organization and coordination of the trial. Hiroki Chikumi and Hideki Nagashima were the chief investigator and responsible for the data analysis. All authors developed the trial design.

Declaration of competing interest

H. Nagashima has received grant support from Astellas Pharma Inc., Asahi Kasei Pharma Co., Daiichi Sankyo Co., Ltd., Pfizer Japan Inc., MSD, K. K., Shionogi & Co., Ltd., Stryker Japan K.K., and Takeda Pharmaceutical Co., Ltd..

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