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Original Article

Metagenomic next-generation sequencing for the diagnosis of oral and maxillofacial space infections

Huan Shi †, Hui Li †, Lingyan Zheng, Wentao Qian, Zhijun Wang, Lisong Xie, Zuoyi Yang, Lingyan Zheng, Changyu Chen, Xiujuan Yang*, Xin Bao **

Department of Oral Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, College of Stomatology, Shanghai Jiao Tong University, National Center for Stomatology, National Clinical Research Center for Oral Diseases, Shanghai Key Laboratory of Stomatology, Shanghai, China

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Read number

Abstract *Background/purpose:* Metagenomic next-generation sequencing (mNGS) has been widely used for the detection of pathogens causing infectious diseases. This study aimed to evaluate the potential ability of mNGS to detect pathogens causing oral and maxillofacial space infection (OMSI) and compare the results with those of the traditional diagnostic microbial culture method.

Materials and methods: We retrospectively reviewed the data of 218 patients diagnosed with OMSI who underwent microbial culture and mNGS at the Department of Oral Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, from July 2020 to January 2022.

Results: The positivity rate of mNGS (216 cases) was significantly higher than that of microbial culture (123 cases). The most frequently detected bacteria were different between these two detection methods. *Streptococcus constellatus* (16.05%, 35), *Streptococcus anginosus* (15.69%, 34) and *Klebsiella pneumoniae* (6.88%, 15) were the most commonly isolated bacteria by culture. However, *Peptostreptococcus stomatis* (61.47%, 134), *Parvimonas micra* (68.35%, 149) and *Streptococcus constellatus* (57.34%, 125) were the most commonly detected bacteria by mNGS. mNGS also has advantages in diagnosing viral infections. The optimal numbers of diagnostic reads were 1162 and 588 for the diagnosis of *Streptococcus anginosus* and *Streptococcus constellatus* infections, respectively. Read numbers were significantly correlated with C-

* Corresponding author.
** Corresponding author.
E-mail addresses: ms_yxj@126.com (X. Yang), baoxin9161@163.com (X. Bao).
† Huan Shi and Hui Li contributed equally to this work.

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reactive protein (CRP), procalcitonin (PCT), and blood glucose levels and neutrophil percentage (NEUT%).
Conclusion: For pathogens causing OMSI, mNGS had a higher rate of microbial pathogen detection and remarkable advantages in identifying coinfections involving viruses and fungi. The read numbers for mNGS are important for diagnostic accuracy and disease severity evaluation.

Introduction

Oral and maxillofacial fascia spaces are continuous and adjacent to one other.[1] These spaces allow the spread of infection, sometimes resulting in severe complications, such as mediastinitis, sepsis and brain abscess, which threaten patients' lives.[2–4] Accurate diagnostic, rapid surgical intervention and pathogen-specific antibiotic therapy are common strategies for the treatment of severe oral and maxillofacial space infection (OMSI). Infections in the oral and maxillofacial regions commonly arise from dentition. Acute pharyngitis/tonsillitis and postsurgical infection account for a lower number of OMSIs. Currently, microbial culture is the gold standard for the detection of pathogens causing OMSI. OMSI is a polymicrobial infection. However, microbial culture has a low positivity rate and limited detection of pathogenic microorganism species.[5]

Metagenomic next-generation sequencing (mNGS) has been widely used in clinical microbial diagnostics because of its high-throughput capacity and fast turnaround time.[6,7] This method allows universal microbial pathogen detection, including detection of viruses, bacteria, fungi, and parasites. More information can be obtained from clinical specimens to improve diagnostic accuracy and help clinicians determine the pathogenesis of a disease.[6,7] However, few studies have reported the use of mNGS in OMSI. In this study, we retrospectively reviewed 218 OMSI cases for which samples were subjected to mNGS and microbial culture detection in our hospital. Herein, we reveal the strengths and limitations of mNGS as a tool for OMSI diagnosis.

Materials and methods

Patients and data collection

From July 2020 to January 2022, a total of 218 patients with diagnosis of OMSI who had been admitted to the Department of Oral Surgery, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, were enrolled. All subjects were enrolled in this study based on the following criteria: computerized tomography (CT)-confirmed OMSI causing swelling and abscess in one or more of the deep facial spaces of the head and neck. The exclusion criteria were as follows: malignant tumor secondary space infection, a history of surgical intervention prior to admission, or pregnancy. Each patient signed an informed consent form. The study was performed in accordance with the Declaration of Helsinki and approved by the Ethical Committee of Shanghai Jiao Tong University School of Medicine Affiliated Ninth People's Hospital (SH9H-2021-T400-2).

Pus samples from all patients included in this study were subjected to pathogen detection by both microbial culture and mNGS. Blood samples were collected within 2 h after admission. The tested serological parameters included routine full and differential blood cell counts (lymphocytes, leukocytes, neutrophils, eosinophils, basophils, and monocytes) and procalcitonin (PCT), blood glucose (at least 4 h after a meal), and C-reactive protein (CRP) levels.

Routine microbial culture

First, pus samples were examined by microscopy with routine laboratory staining and culture of bacteria, fungi, and mycobacteria. The samples were subjected to our hospital protocol for microbial culture. The pus was diluted and placed onto a blood agar plate. After that, the samples were cultured in aerobic environments at 37 °C. Then, the samples were examined after 24 h.

Metagenomic next-generation sequencing and analysis

Nucleic acid extraction, library preparation, and sequencing

A QIAamp® UCP Pathogen DNA Kit (Qiagen, Hilden, German) was used to extract DNA according to the manufacturer's instructions. The kit was used for DNA extraction from the pus, which was originally developed for bacteria. However, recent studies using bronchoalveolar lavage fluid (BALF) have also shown that it can be used to extract viral DNA.[8] DNA libraries were constructed by using a Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). An Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) was used for quality control of the DNA libraries. A Qubit dsDNA HS Assay Kit (Agilent) and High Sensitivity DNA Kit (Agilent) were used for quality assessment. Library pools were subsequently loaded on an Illumina Nextseq 550Dx platform (Illumina) for 75 cycles of single-end sequencing. Each library generated at least 20 million reads. Peripheral blood mononuclear cell (PBMC) samples from healthy donors were used as negative controls with the same protocol.

Bioinformatic analysis

Adapter contamination, duplicate reads, low-quality reads and short reads (<50 bp) were removed by Trimmomatic.[9]

Then, human sequence data mapped to a human reference genome (hg38) were identified and excluded using Burrows–Wheeler Aligner software.

After alignment, a list of suspected microorganisms was obtained by comprehensively evaluating multiple indicators. Microbiota composition profiles were inferred from quality-filtered forward reads using Kraken V.2.1.2 and Bracken V.2.6.2. The microorganism genome database contained the genomes or scaffolds of 9694 bacteria, 1551 fungi, 6760 viruses, and 305 parasites.

Statistical analysis

Pearson correlation analysis was used when the dual variables were normally distributed, and Spearman correlation analysis was used when these were not normally distributed. To evaluate the diagnostic efficiency of mNGS, receiver operating characteristic (ROC) curve analysis was used to calculate the area under the curve (AUC). The cutoff values of the read values were determined in line with the maximum Youden index. The values range from 0 to 1; values closer to 1 indicate a higher diagnostic value. Data analysis was conducted using SPSS 16.0 software.

Results

Patient characteristics and clinical features

A total of 218 patients, including 151 males (69.27%) and 67 females (30.73%), were enrolled in this study. The mean age was 53.72 ± 17.40 years. Among the 151 males, 56 (37.08%) patients were aged ≥60 years. The most common cause of OMSI was odontogenic infection (83.94%, 183/218), followed by peritonsillar abscess (5.50%, 12/218) and postsurgical infection (3.67%, 8/218). Only a small percentage of OMSI (6.88%, 15/218) was derived from parotitis, lymphadenitis or sialadenitis. Surgical intervention was performed under local anesthesia in 126 (57.80%) patients with moderate OMSI. Patients with severe or extremely severe OMSI or with a poor general condition underwent surgery under general anesthesia. The mortality rate in the 218 patients in this study was 1.38% (3). Laboratory test results together with the above data are shown in Table 1. Hyperglycemia was observed in 104 patients. Among them, 39 patients claimed a poorly controlled diabetes history. Fasting blood glucose (mmol/L) was found to be ≥ 20 in 8 patients, ≥ 11 < 20 in 29 patients and ≥7 < 11 in 67 patients.

Comparison of mNGS and microbial culture pathogen detection

Samples from all 218 patients were subjected to both mNGS and microbial culture. The positivity rate of mNGS was 99.08% (216), which was significantly higher than that of routine culture (56.42%, 123). The distribution of pathogens identified by microbial culture and mNGS for all 218 patients is shown in Fig. 1. *Streptococcus constellatus* (16.05%, 35), *Streptococcus anginosus* (15.60%, 34) and *Klebsiella pneumoniae* (6.88%, 15) were the most commonly isolated bacteria by culture. However, *Peptostreptococcus stomatis* (61.47%, 134), *Parvimonas micra* (68.35%, 149) and *Streptococcus constellatus* (57.34%, 125) were the most commonly detected bacteria by mNGS. Interestingly, microbial culture did not detect a single isolate of *P. stomatis* or *P. micra*. *Porphyromonas endodontalis* and *Prevotella oris* also had high positivity rates by mNGS. *Candida albicans* was detected by both mNGS and microbial culture. Viral pathogens, including *Torque teno virus* (TTV) (26.15%, 57) and *human alphaherpesvirus* (6.88%, 15), were identified by only mNGS. Moreover, 33 (57.89%) patients with TTV underwent surgery under general anesthesia. The rate of receiving general anesthesia in patients without TTV was 36.00%. The results indicated that patients with TTV suffered more severe infection.

Among the 218 specimens, dual positivity on mNGS and culture was observed in 122 (55.96%) cases, and dual negativity was observed in only 1 (0.46%) case. In total, 94 (43.12%) cases were positive on only mNGS, and 1 (0.46%) case was positive on only microbial culture. Among the 122 dual-positive cases, 72 (59.02%) had completely matched results, while 50 (40.98%) had mismatched results (Fig. 2).

Diagnostic value of the number of reads in the mNGS results

Read counts have an important role in the interpretation of mNGS results. The bacteria detected by mNGS and microbial culture (*Streptococcus constellatus, Streptococcus anginosus* and *K. pneumoniae*) with the largest numbers of matches were chosen for analysis. We performed ROC curve analysis to determine the diagnostic value of the number of reads in the mNGS results and found the optimal number of reads for diagnosis. The AUC values revealed unsatisfactory performance of the number of reads for the detection of *Streptococcus constellatus* (AUC: 0.579) and *Streptococcus anginosus* (AUC: 0.592). The optimal numbers of diagnostic

Table 1 Demographic characteristics of included patients (n = 218).

Characteristics	Value
Age (year, mean ± SD)	53.72 ± 17.4
Gender (Female/Male, n)	67/151
Etiology (n, %)	
Odontogenic Abscess	183 (83.94)
Peritonsillar Abscess	12 (5.5)
Post-surgery Infection	8 (3.67)
Others	15 (6.88)
Laboratory test (mean ± SD)	
WBC (10^9/L)	14.42 ± 11.99
Total amount of neutrophils (10^9/L)	11.28 ± 5.54
Ratio of neutrophils (%)	79.95 ± 10.95
CRP (mg/dl)	77.21 ± 77.47
PCT (ng/L)	1.26 ± 3.73
Blood glucose (mmol/L)	8.62 ± 4.44
Mortality (n, %)	3, 1.3%
Local anesthesia/General anesthesia (n)	126/92

WBC white blood cell, CRP C-reactive protein, PCT procalcitonin, NEUT neutrophil, SD standard deviation.

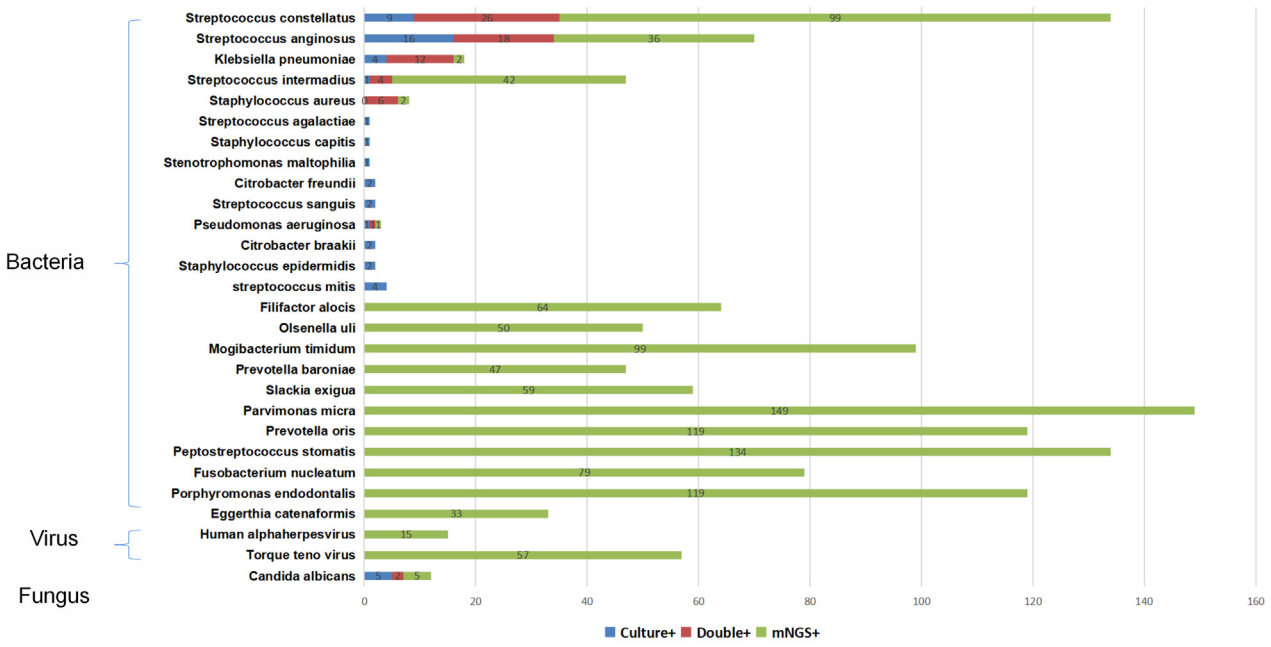

Figure 1 Distribution of positive and matched results of metagenomic next-generation sequencing (mNGS) and routine microbial culture according to the detected pathogenic species. The number of patients who were detected the pathogenic species has been written in barchart.

reads were 588 (specificity: 0.385, sensitivity: 0.846) and 1162 (specificity: 0.529, sensitivity: 0.722) for *Streptococcus constellatus* and *Streptococcus anginosus*, respectively (Fig. 3A and B). However, the correlation analysis results showed that the read numbers for *Streptococcus anginosus* and *K. pneumoniae* were positively correlated with CRP levels ($r = 0.5807$, $P = 0.0091$; $r = 0.5944$, $p = 0.0457$) (Fig. 3C and D) (Table 2). The read number for *Streptococcus constellatus* was positively correlated with PCT and blood glucose levels and NEUT% (Fig. 3E–G) (Table 2).

Discussion

Recently, with the advancement of mNGS technologies, the costs of mNGS have decreased. A rapid reporting time, comprehensive databases and a variety of data analysis tools have allowed the use of mNGS in clinical pathogen diagnostics. The application of mNGS in a large number of OMSI patients has never been reported before. In this study, we conducted a comprehensive analysis of the diagnostic performance of mNGS for pathogen detection in 218 OMSI patients. To our knowledge, this is the first study to describe the application of mNGS in OMSI patients and to compare its diagnostic value with that of routine microbial culture.

In accordance with a previous study, we found that elderly male patients accounted for the majority of OMSI cases.[10] Odontogenic infection (83.94%) was the main etiology of OMSI in patients in our hospital. Three of the 218 patients did not survive an extremely severe case of OMSI. All patients received rapid surgical intervention and empirical antibiotic treatment at admission based on signs of infection in routine blood tests. Almost all patients had elevated white blood cell and neutrophil counts together with increased blood CRP and PCT levels. Hyperglycemia

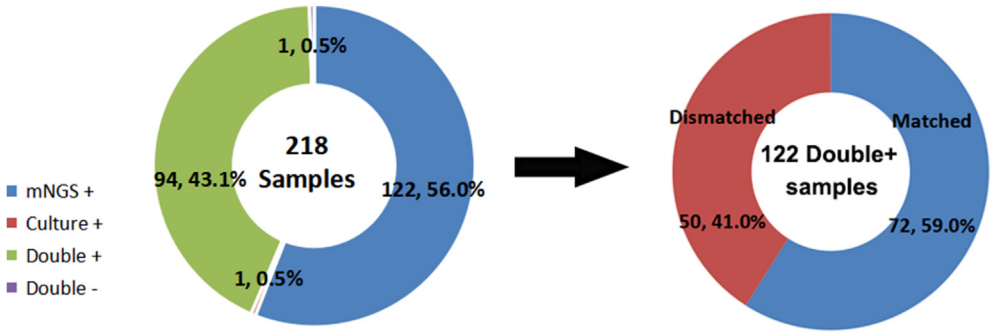

Figure 2 Culture and mNGS showed double-positive results for 122 (55.96%) specimens, in which 72 (59.02%) cases were completely matched, while a mismatch was observed in 50 (40.98%) cases.

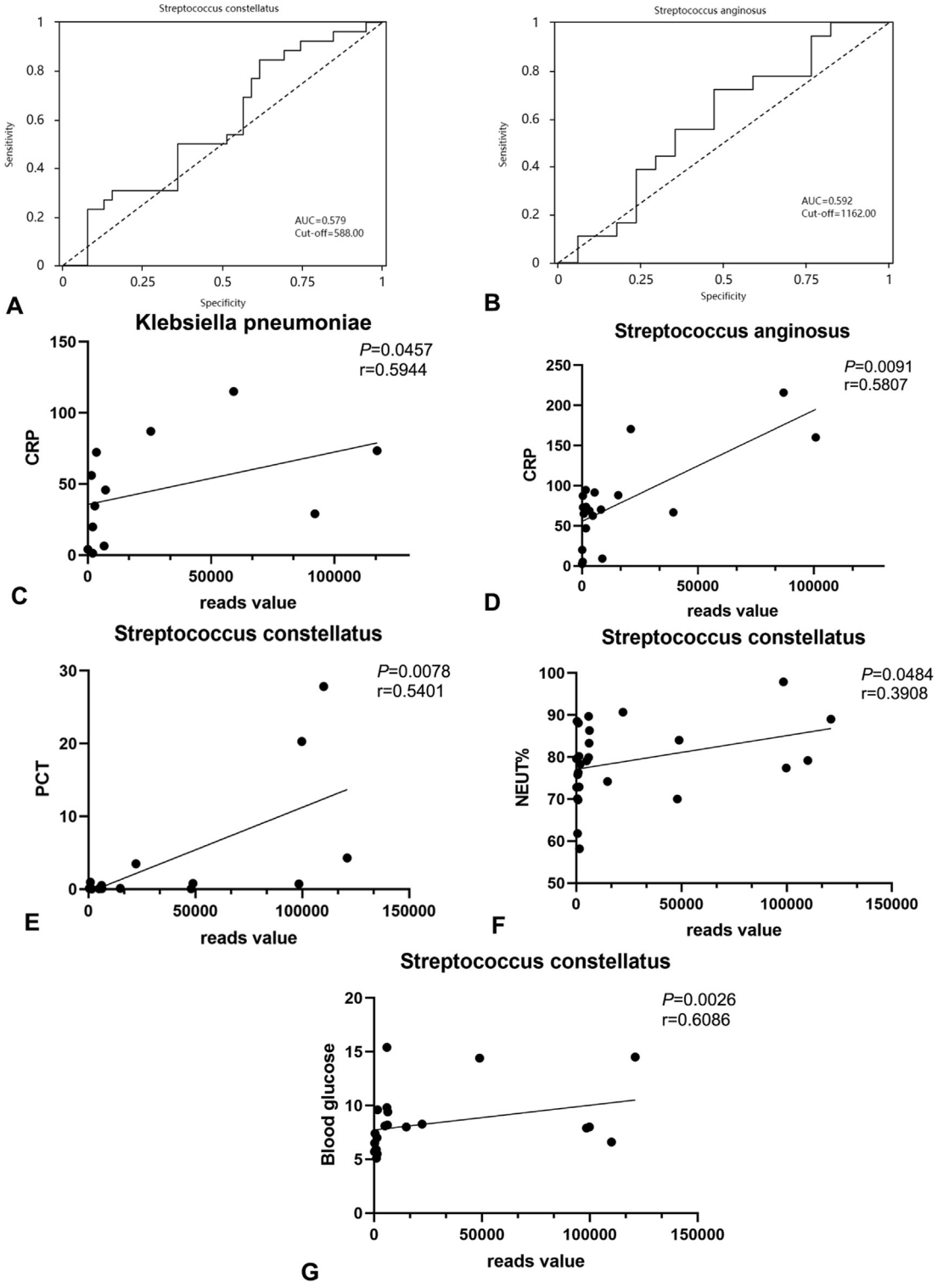

Figure 3 mNGS reads for diagnosis. A. ROC analysis of read numbers for *Streptococcus constellatus;* B. ROC analysis of read numbers for *Streptococcus anginosus*; C. correlation analysis between read numbers and CRP levels for *Klebsiella pneumoniae*; D. correlation analysis between read numbers and CRP levels for *Streptococcus anginosus*; E. correlation analysis between read numbers and PCT levels for *Streptococcus constellatus*; F. correlation analysis between read numbers and NEUT% for *Streptococcus constellatus*; G. correlation analysis between read numbers and blood glucose levels for *Streptococcus constellatus.*

Table 2 Correlation analysis results of reads number and laboratory test.

	Streptococcus constellatus		*Streptococcus anginosus*		*Klebsiella pneumoniae*	
	P	r	*P*	r	*P*	r
WBC	0.290	0.216	0.637	−0.116	0.276	−0.343
CRP	0.366	0.193	0.009**	0.581	0.216	−0.385
NEUT	0.183	0.270	0.721	−0.088	0.042*	0.594
NEUT%	0.048*	0.391	0.147	0.346	0.633	−0.154
PCT	0.008**	0.540	0.081	0.410	0.503	0.214
Blood glucose	0.003**	0.609	0.336	0.234	0.829	−0.070

WBC white blood cell, CRP C-reactive protein, PCT procalcitonin, NEUT neutrophil, *$P < 0.05$, **$P < 0.01$.

was also commonly observed in OMSI patients. Stress hyperglycemia was observed in partial OMSI patients without a diabetes history.[11] However, most OMSI patients with hyperglycemia had an uncontrolled diabetes history. Uncontrolled diabetes mellitus has been correlated with the occurrence of OMSI and is a high-risk factor promoting severe complications.[12,13]

Undoubtedly, mNGS had a higher pathogen positivity rate than routine microbial culture. The diagnostic sensitivity has been widely reported as an advantage of mNGS.[6] In this study, we also found that there was an obvious difference in the detected bacterial species between mNGS and microbial culture. Most species detected by microbial culture were aerobic or facultative anaerobic organisms. Anaerobic organisms, including *P. micra*, *P. endodontalis*, *P. stomatis* and *Prevotella* spp., which were the most frequently detected pathogens by mNGS, were rarely observed in our routine microbial culture results. This result indicates that because of the stringent culture environment, poor anaerobic organism detection is a disadvantage of routine microbial culture. In previous studies, *Prevotella*, *Porphyromonas* and *Peptostreptococcus* species is the most detected anaerobic organisms. Böttger et al. compared molecular method detecting pathogen of odontogenic abscesses with cultural bacterial determination carried out in the clinical routine of a hospital. The results revealed that routine clinical culture probably only provides a results of irreality and should be supplemented by molecular methods in the future.[14] *C. albicans* is a typical opportunistic pathogen that causes oral infectious diseases. Under normal conditions, *C. albicans* colonization is limited.[15] *C. albicans* proliferation usually indicates suppression of the host's immune system. We do not think the *C. albicans* detection results are directly related to OMSI but rather are more likely due to the changes in the host immune response secondary to OMSI. However, *C. albicans* infection undoubtedly indicated extremely severe OMSI. The positive rates of fungus identification were similar between the two diagnostic methods, but this result was different from those of previous studies that reported that mNGS had a low positivity rate for fungal detection.[16,17] The samples used for mNGS in the above two studies were plasma, and the results were compared with routine blood culture. In our study, pus was collected to conduct mNGS analysis and routine bacterial culture. Different specimens may contribute to the different results. Further studies are needed to explain this phenomenon. A total of 57 patients were found to have TTV infection, according to mNGS. TTV is a commensal human virus, and the prevalence of TTV in human populations may reach 100%.[18] Data on TTV prevalence rates and genetic heterogeneity between healthy controls and patients with various pathologies are abundant. Rocchi et al. reported that TTV can activate the immune response through TLR9.[19] However, TTV infection in OMSI has never been reported before. We notably found that patients with TTV were suffering more severe OMSI. Similar results were found in septic shock patients with TTV infection.[20] However, no direct association was found between TTV infection and OMSI. Further studies are still needed to discover its potential role in OMSI.

It has been reported that the read number in mNGS can be used for the interpretation of distinct pathogenic infections.[21–23] In this study, we analyzed the diagnostic performance of read numbers for the two most frequently detected pathogens based on the results of microbial culture. The optimal cutoff read numbers can help clinicians avoid false-positive reports in mNGS. However, the performance for these pathogens was not sufficient. The optimal cutoff read numbers for *Streptococcus constellatus* and *Streptococcus anginosus* were 588 and 1162, respectively. The sensitivity was acceptable, but the specificity was unsatisfactory. However, the correlation analysis showed that the read number for these bacteria was positively correlated with several laboratory test results. CRP and PCT have long been considered indicators of the severity of an infectious disease, including OMSI. Therefore, the above results suggest that the diagnostic value of read number as a marker deserves more attention. Further studies including larger sample sizes are needed to verify the present results.

This study initially reported the usage of mNGS for pathogen detection in OMSI patients. Higher positivity rates, especially for anaerobic organisms and viruses, are advantages of mNGS in comparison with routine microbial culture. We also first identified the optimal cutoff reads for diagnosing the two most commonly detected pathogens, *Streptococcus constellatus* and *Streptococcus anginosus*, in OMSI patients, which is favorable for the clinical application of mNGS. With the progress of mNGS-applied research, mNGS could be a promising diagnostic method for pathogen detection.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2022.09.002.

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