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Identification of novel bacterial species in the blood of patients with neonatal sepsis

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Abstract

Background The clinical diagnosis of neonatal sepsis remains difficult because of various challenges, such as culturing the bacteria and avoiding contamination. Therefore, this study aimed to identify bacterial pathogens in patients with clinically diagnosed neonatal sepsis by next-generation sequencing (NGS).

Methods High-throughput NGS and traditional culture identification were performed by comparing samples from newborns with neonatal sepsis with healthy control infants. All blood samples were separately inoculated into anaerobic and aerobic bottles and incubated for 7 days at 37 °C, the positive specimens were then identified. Novel bacteria identified through high-throughput NGS were analysed using polymerase chain reaction (PCR), PCR products were verified by Sanger sequencing. Wilcoxon rank-sum and chi-square tests were performed to assess the significance of differences in species abundance between groups. Subjects were clinically diagnosed and hospitalized at the Pediatrics Department of Shenzhen Seventh People's Hospital and Pediatrics Department of the Longhua Branch of Shenzhen People's Hospital. Experiments were performed at the Shenzhen Seventh People's Hospital. The experimental group comprised 45 newborns clinically diagnosed with neonatal sepsis (age: 0–28 days; 28 males, 17 females). Fifteen normal newborns aged 0–28 days (7 males, 8 females) were included as the control group.

Results High-throughput NGS showed a positivity rate of 44% (20/45) for bacteria in patients clinically diagnosed with neonatal sepsis, whereas traditional bacterial culture identification showed a positivity rate of 0% (0/45). The four main bacterial species identified were *Anoxybacillus kestanbolensis*, *Geobacillus vulcani*, *Klebsiella oxytoca*, and *Acinetobacter guillouiae*.

Conclusions *A. kestanbolensis*, *G. vulcani*, *K. oxytoca*, and *A. guillouiae*, newly discovered bacteria in patients with neonatal sepsis, were identified with high-throughput NGS. Which may result from maternal intrauterine infection or birth-canal infection and have a high clinical-cure rate. Owing to a lack of methods to culture these bacteria, their role in neonatal sepsis remains unclear. A definite diagnosis cannot rely solely on bacterial culture identification for patients with a suspected diagnosis and clinical diagnosis of neonatal sepsis and should involve other effective diagnostic techniques.

Keywords Diagnosis, Newborns, Next-generation sequencing, Neonatal sepsis

Background

Blood infections remain a significant cause of mortality in neonatal hospital units. Neonatal sepsis (NS) is a bacterial infection of the blood in newborns with a high incidence and mortality; the annual mortality is 25–30% per 3 million patients [1]. The clinical diagnosis of NS is difficult; therefore, continuous patient observation is important [2]. The current diagnostic methods mainly include traditional culture methods and nonculture-based

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technologies, including molecular methods and mass spectrometry; cell adhesion molecules, haematological indicators, interleukins, and acute-phase reactants are also used as biomarkers for the diagnosis of NS [3]. However, blood culture remains the current gold standard for NS diagnosis. But it is affected by several factors, including bacterial contamination from the skin surface during blood collection, small sample volumes, site of blood collection, time of blood collection, and use of antibiotics. In some cases, these factors can lead to false-positive or false-negative results. Blood culture is unsuitable for rare bacterial pathogens or those with stringent requirements for specific nutrients and culture conditions, and the cultivation time is long. To overcome these limitations, employing molecular biology techniques may be useful owing to the rapidity and the small sample size required for analysis. In this study, high-throughput next-generation sequencing (NGS) was combined with traditional blood culture to screen for bacterial pathogens in patients clinically diagnosed with NS, and polymerase chain reaction (PCR) amplification was performed using specific primers for bacterial pathogens followed by verification using Sanger sequencing.

Methods

Patient selection

Forty-five hospitalized newborns clinically diagnosed with NS and 15 normal newborns in the Pediatrics Department of Shenzhen Seventh People's Hospital, and Pediatrics Department of the Longhua Branch of Shenzhen People's Hospital from October 2017 to October 2022, were included in this study. The subjects were aged between 0 and 28 days and comprised 28 males and 17 females. Fifteen normal newborns aged between 0 and 28 days, comprising 7 males and 8 females, were included as the control group. This study is retrospective. The subject inclusion criteria were in accordance with the Protocol for Diagnosis and Treatment of Neonatal Septicemia approved by the Subspecialty Group of Neonatology, Pediatric Society, Chinese Medical Association, and Editorial Committee of the *Chinese Journal of Pediatrics* [4] (see Additional file 1). The exclusion criteria for the normal newborn control group included the presence of inflammation, infection, or any clinical symptom as determined by the Chinese Medical Association. The three main diseases in patients with NS and their corresponding treatments in the 45 subjects with NS are shown in Additional file 2. A combination of these three diseases was observed in patients with NS.

Collection and processing of samples

Blood samples were collected immediately following hospitalization or birth of the subjects but before the administration of antibiotics. After cleaning and disinfecting the skin with alcohol for 1 min, a 1-mL blood sample was collected aseptically into a routine blood collection tube for high-throughput NGS, and 2 mL of venous blood was collected for traditional bacterial culture identification (1 mL each for anaerobic and aerobic culture).

Blood culture and isolate collection

Blood samples were separately placed in anaerobic and aerobic bottles and incubated at 35 °C in a BACT/ALERT 30 automated blood culture system (bioMerieux, Marcy l'Etoile, France) for 7 days or until microbial growth was observed; after which, the blood samples were transferred onto blood nutrient agar for further isolation and culture. Bacterial morphology was observed using gram staining followed by microscopic analysis. Pure bacterial isolates were identified using a Microflex LT/SH mass spectrometer (Bruker Daltonics, Hamburg, Germany).

DNA extraction and library construction

Bacterial DNA was extracted from routine blood collection samples of the 60 newborns (45 experimental [NS] and 15 control) who were not treated with antibiotics as previously described by Wu et al., and DNA concentration and integrity were checked [5]. Using qualified DNA extracts, the V4 region of the 16S rRNA gene and the internal transcribed spacer 1 (ITS1) region of the ITS sequence were amplified using primers (see Additional file 3). Double-indexed fusion primers containing sequencing adapters were synthesized as described by Wu et al. [5].

Sequencing and de novo assembly

After completion of the isolate collection, sequencing libraries were successfully constructed for 20 blood samples from the NS group and subjected to high-throughput NGS. The quality control (Q30) was set to ≤ 80%. PCR was performed using the Agilent 2100 platform (Agilent, Santa Clara, CA, USA); water was used as a negative control. Illumina sequencing data were filtered, and high-quality reads were assembled into tags clustered into operational taxonomic units (OTUs) at 97% similarity. OTUs were compared with the Greengene and UNITE databases for species annotation. Differences in selected species between NS and healthy control groups were tested for significance. Sequences were assembled using FLASH (v1.2.11) with a minimum overlap of 15 base pairs (bp) and a mismatch ratio of the overlap region of 10%. Reads that did not overlap with other sequencing reads were excluded from the analysis.

Genomic taxonomy classification

Tags were clustered into OTUs at 97% similarity using USEARCH (v7.0.1090), and a representative sequence of

each OTU was chosen for comparison with the Greengene_2013_5_99 database using RDP classifier (v2.2) for species annotation at a confidence threshold of 0.5. By comparison with the database, OTUs were classified at six taxonomic levels (phylum, class, order, family, genus, and species), and histograms for each sample's taxonomic profiling were constructed. At the class, order, family, and genus levels, all OTUs with an abundance of less than 0.5% in a single sample were pooled as 'others', whereas at the species level, all OTUs with an abundance of less than 3% in a single sample were pooled as 'others'.

Identification of novel species genomes

Hierarchical clustering heatmaps were generated for all OTUs at the phylum, class, order, family, genus, and species levels following the method of species annotation illustrated in the genomic taxonomy classification above. Heatmap analysis was performed on the relative abundance of each OTU in each sample. Plots were generated using the gplots package in R (v3.1.1) based on Euclidean distances and complete clustering. The alpha diversity of the samples was calculated using Mothur (v1.31.2), and the corresponding rarefaction curve was generated using R (v3.1.1). Sample clustering was conducted using the unweighted pair group method with arithmetic mean (UPGMA). The Wilcoxon rank-sum test was performed in R to analyse the significance of between-group differences. All *p*-values were adjusted using the Benjamini–Hochberg method with the command p.adjust in R (v3.1.1).

Molecular identification of bacterial DNA

Specific gene sequences

Based on the high-throughput NGS results, the four species with the highest OTU abundance, namely, *Anoxybacillus kestanbolensis*, *Geobacillus vulcani*, *Klebsiella oxytoca*, and *Acinetobacter guillouiae*, were selected. The complete genome sequences of these species were retrieved from the EMBL and GenBank databases and used for specific primer design using the Primer 6.0 software (PREMIER Biosoft, Palo Alto, CA, USA). For each strain, two nucleotide sequences showing significant differences were selected to design two pairs of specific primers (BGI, Guangzhou, China). The specific primers are listed in Additional file 4.

PCR amplification and electrophoresis

Positive samples were amplified by PCR using the following reaction steps: initial denaturation at 96 °C for 5 min, 34 cycles of denaturation at 96 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The PCR products were detected using electrophoresis on a 1% agarose gel and stored at 4 °C until further analysis.

Sanger sequencing

PCR products showing a clear single band on the electrophoresis gel were subjected to Sanger sequencing using a type ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing results were compared with the data from EMBL and GenBank databases to identify the bacterial species using the BLAST algorithm.

Statistical analysis

As described by Wu et al. [5], Wilcoxon rank-sum and chi-square tests were used to assess the significance of between-group differences in species abundance. Data processing was performed using bioinformatics software FLASH (v1.2.11), USEARCH (v7.0.1090), RDP classifier (v2.2), R (v3.1.1), and Mothur (v1.31.2), with $p < 0.05$ indicating a significant difference.

Results

Distribution of pathogens

The positivity rate of high-throughput NGS was 44% (20/45), the negativity rate was 56% (25/45), and the positivity rate of traditional bacterial culture was 0% (0/45); the negativity rate was 100% (45/45), respectively, with a significant difference between high-throughput NGS and traditional bacterial culture positive groups ($\chi^2 = 16.92$, $p < 0.01$) and no significant difference between high-throughput NGS and traditional bacterial culture negative groups ($\chi^2 = 3.265$, $p > 0.01$). In the healthy control group, the traditional bacterial culture results were all negative, and NGS detected very low levels of bacterial DNA in the blood.

Genomic classification and identification of novel genome species

A total of 818,514 tags were generated from the 20 samples, and 1390 OTUs were obtained by clustering the tags. OTUs were classified into 29 phyla (see Additional file 5), 9 classes (see Additional file 6), 12 orders (see Additional file 7), 12 families (see Additional file 8), 10 genera (Fig. 1), and 4 species (Fig. 2) by comparison with the Greengene database. The main bacterial species at the phylum level were Proteobacteria, Firmicutes, Thermi, and Bacteroides. The main bacteria at the class level were Gammaproteobacteria and Bacilli. The main bacteria at the order level were Enterobacterales, Bacillales, and Pseudomonadales. The main bacteria at the family level were Enterobacteriaceae, Bacillaceae, and Moraxellaceae. The main bacteria at the genus level were *Anoxybacillus*, *Bacillus*, and *Klebsiella*. The main bacteria

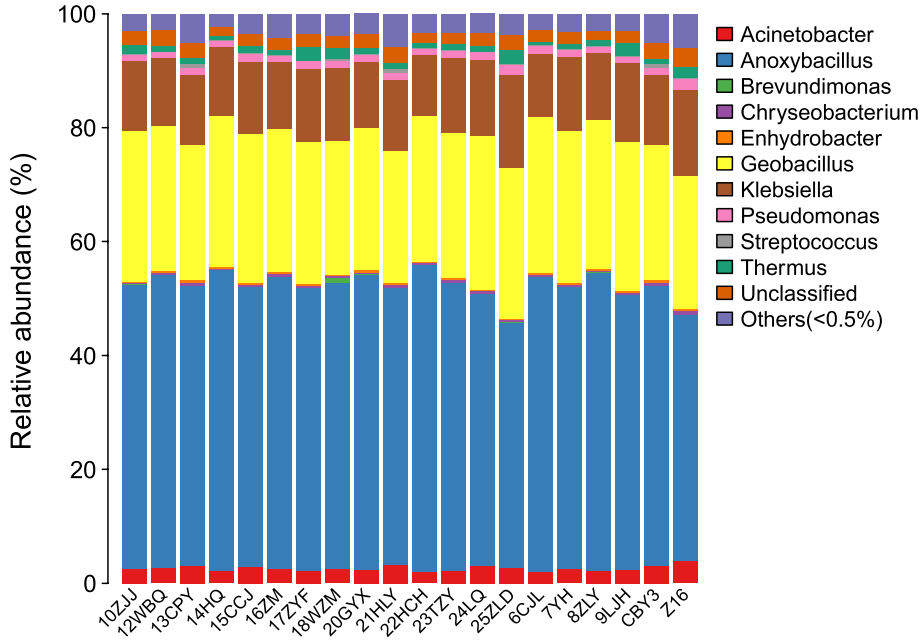

Fig. 1 Histogram of bacterial composition at the genus level

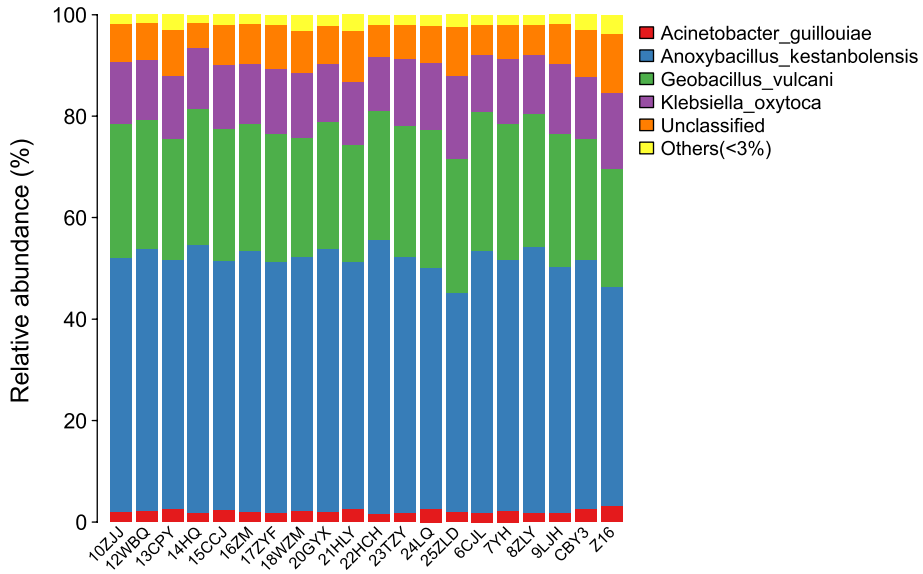

Fig. 2 Histogram of bacterial composition at the species level

at the species level were *G. vulcani*, *A. kestanbolensis*, *A. guillouiae*, and *K. oxytoca*.

Heatmap analysis

Heatmap clustering of bacterial OTUs was performed at the six taxonomic levels of phylum, class, order, family, genus, and species. The main OTUs with the highest similarity in relative abundance across samples at the phylum level belonged to Proteobacteria, Firmicutes, Thermi, and Bacteroides. The main OTUs at the class level belonged to Gammaproteobacteria and Bacilli. The main OTUs at the order level belonged to Enterobacterales, Bacillales, and Pseudomonadales. The main OTUs at the family level belonged to Enterobacteriaceae, Bacillaceae, and Moraxellaceae. The main OTUs at the genus level belonged to *Anoxybacillus*, *Klebsiella*, and *Geobacillus* (Fig. 3). The main OTUs at

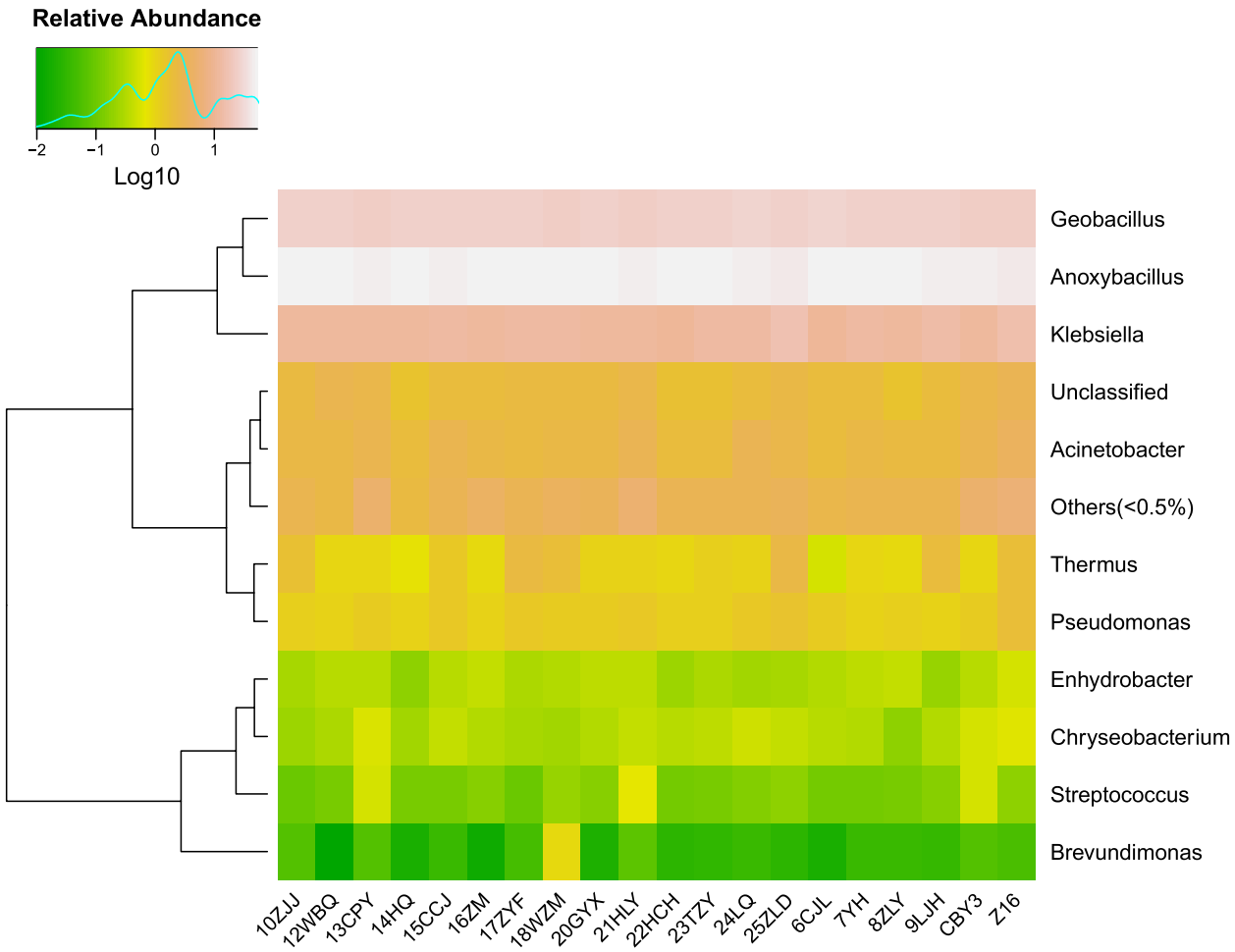

Fig. 3 Cluster heat map of bacterial abundance at the genus level

the species level were *G. vulcani*, *A. kestanbolensis*, *A. guillouiae*, and *K. oxytoca* (Fig. 4).

Alpha-diversity analysis and sample clustering based on bacterial composition

Alpha-diversity analysis showed that multiple species were present in each sample. The alpha-diversity rarefaction curves of the samples showed that the sequencing depth was sufficient to capture the entire bacterial diversity in the samples (see Additional file 9). UPGMA clustering, which indicates the bacterial composition of samples, showed that similar species existed in all 20 NGS-positive samples (see Additional file 10).

Electrophoresis and sequencing of PCR products specific to 4 bacterial species

PCR amplification performed on infant blood demonstrated that using two pairs of specific primers for the four bacterial species of *A. kestanbolensis*, *G. vulcani*, *K. oxytoca*, and *Acinetobacter guillouiae* yielded DNA sequences of 388 (372 bp), 213 (222 bp), 360 (306 bp), and 340 (840 bp) nucleotides in length (see Additional file 11), respectively. Sanger sequencing results were consistent with those of NGS (see Additional files 12, 13, 14, 15, 16, 17, 18 and 19).

Discussion

Monitoring the incidence, prognosis, and development of NS requires robust methods while considering the evolving clinical definitions of sepsis [6]. There is currently no international consensus for defining NS. In 2016, the *Journal of the American Medical Association* named this disease 'SEPSIS-3', which is a life-threatening disease characterized by the host's dysregulated response to infection and consequent organ dysfunction [7]. NS diagnosis is divided into three categories: suspected diagnosis, clinical diagnosis, and definite diagnosis [4]. Given that newborns are the most susceptible to sepsis, it is necessary to understand the associated risk and etiological factors for better NS management.

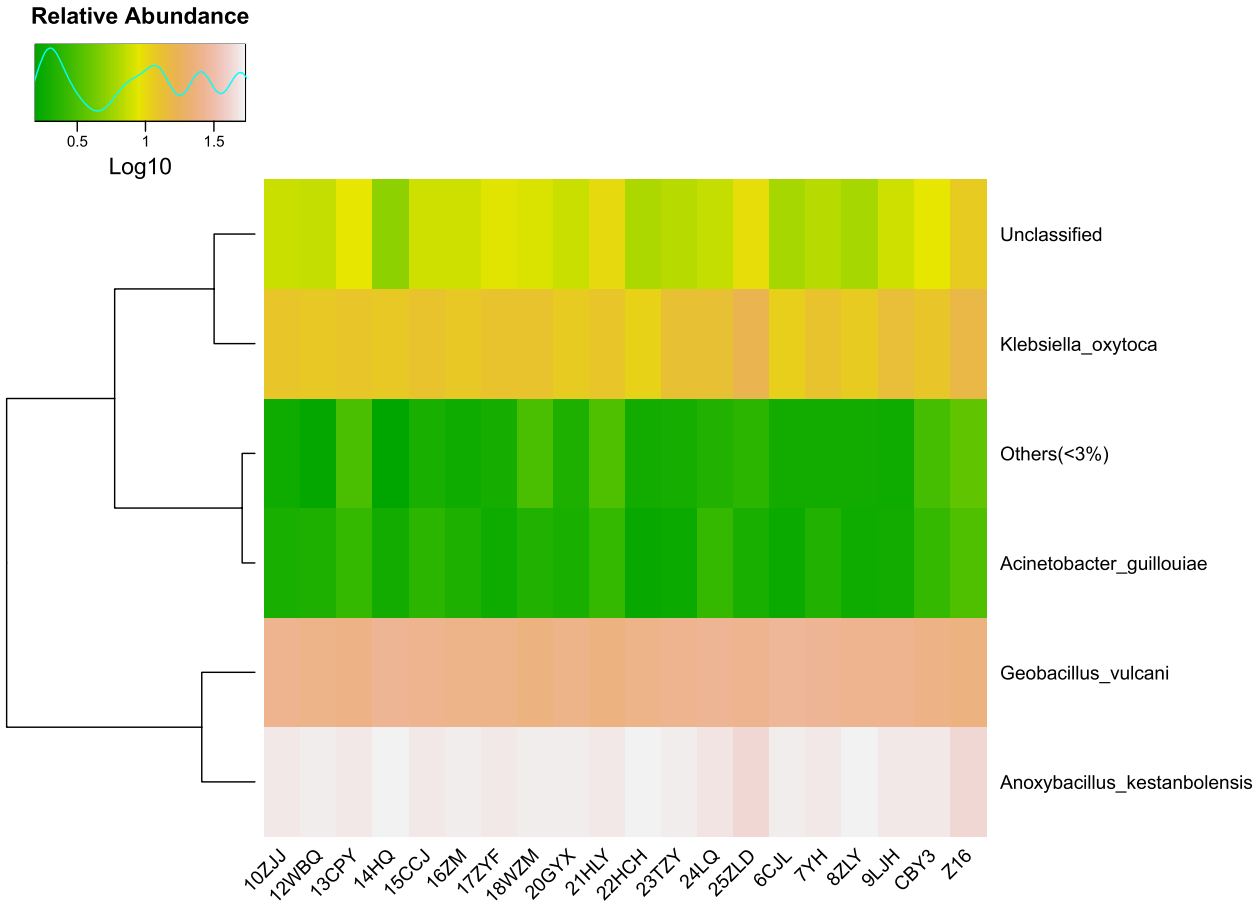

Fig. 4 Cluster heat map of bacterial abundance at the species level

The neonatal immune system is vulnerable due to characteristics including decreased cellular activity, underdeveloped complement systems, preferential anti-inflammatory responses, and insufficient pathogenic memory [8]. Neonatal susceptibility is caused by an immature immune system, maternal infection, adverse environment, and other risk factors. Additionally, studies have shown that the IL-1β rs1143643 G > A gene polymorphism is related to the incidence of NS [9]. Researchers have suggested that NS increases the risk of neurodevelopmental disorders in premature infants [10]. Given that an early diagnosis of NS can improve prognosis, it is critical to identify novel biomarkers for NS. As biomarkers of neonatal sepsis, the most commonly used laboratory parameters include whole blood cell count, C-reactive protein, and procalcitonin, all of which have significant limitations in sensitivity and specificity. Other serum biomarkers, including interleukin-6, presepsin, cluster of differentiation (CD) 64, CD11b, serum amyloid A, S100 protein A12, lipopolysaccharide-binding protein, volatile organic compounds, and soluble triggering receptor expressed on myeloid cell-1, have also been used for the identification of neonatal sepsis [11]. Expert studies have found that CRP levels are associated with bacteremia, fungemia, and viremia detected by blood mNGS [12]. In this study, we found that the total number of WBCs or CRP in the vast majority of NGS-positive newborns with NS were elevated. Progranulin is a promising biomarker for the diagnosis of EOS, as its use combined with the detection of procalcitonin can improve the diagnosis of sepsis [13]. Studies have shown that although blood culture results are similar to PCR results in EOS, the positivity rate of PCR is significantly higher than that of blood culture, with 16S rRNA PCR showing higher sensitivity and allowing for a rapid and accurate diagnosis [14].

Blood culture is the most suitable method for antimicrobial susceptibility testing [14]. İstanbullu et al. point out that the 16S rRNA genes of all suspected microorganisms should be subjected to real-time PCR detection to improve the diagnostic sensitivity of NS [15]. In the current study, we amplified the 16S rRNA gene and ITS sequence in patient samples to construct gene libraries and performed high-throughput NGS analysis, covering

the genes of prokaryotic and eukaryotic microorganisms, and neither viruses nor fungi were detected. Akbarian-Rad et al. found that NS is highly prevalent in patients suspected of having NS, indicating the importance of taking preventive measures, conducting routine evaluation, and closely monitoring newborns [16]. Zhou et al. found through a retrospective study that the detection rate of pathogenic microorganisms in blood mNGS was significantly higher than that of pathogens in blood culture. Among all the positive bacterial and fungal detection results, only 12.00% of blood mNGS were consistent with blood culture, suggesting that mNGS could not completely replace blood culture in patients with suspected blood infection [12]. Herein, among the patients clinically diagnosed with NS, 44% were definitively diagnosed using high-throughput NGS. The discrepancy between the clinical diagnosis and final diagnosis in traditional bacterial culture methods indicates that this method alone cannot be used to confirm a suspected diagnosis or clinical diagnosis of NS; thus, other effective and practical diagnostic techniques must be used.

The pathogens responsible for NS vary worldwide; the most common pathogens in developing countries are gram-negative bacteria, including *Acinetobacter* spp., *Klebsiella* spp., and *Escherichia coli*. Bacterial culture-based studies have confirmed that *Streptococcus pneumoniae* is the most common pathogen responsible for NS in South China [17]. *S. pneumoniae* is primarily associated with LOS in premature infants, whereas group B *Streptococcus* is the main EOS pathogen. *E. coli* is common in both LOS and EOS and leads to the highest mortality rate [18]. El-Amir et al. reported that EOS is primarily caused by E. coli, whereas LOS is caused by *Staphylococcus aureus* [14]. Margaret et al. found that group B streptococci and *E. coli* were the most common in EOS, while coagulase-negative *Staphylococcus* was the main cause of LOS [8]. The herpes simplex virus may also cause potentially devastating infections in newborns [19]. Gao et al. found that the positivity rate of gram-negative bacteria in NS exceeds that of gram-positive bacteria and fungi, and that the four most dominant bacterial pathogens in NS are *E. coli* (21.9%), *Klebsiella pneumoniae* (21.9%), group B *Streptococcus* (13.2%), and *S. aureus* (6.8%). *Enterobacter* spp. and *K. pneumoniae* are considered the main bacterial pathogens responsible for NS in Iran. Moreover, 22.9% of *Enterobacter* spp. responsible for NS are extended-spectrum beta-lactamase-producing bacteria [20]. Extended-spectrum beta-lactamase-producing multidrug-resistant *E. coli* have emerged as the main pathogens responsible for early NS, particularly in premature infants [21]. Common bacterial pathogens responsible for sepsis in surgery are *Enterococcus faecalis*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., which are similar to the common bacterial pathogens in NS [22].

The bacterial species detected in this study included *Streptococcus* spp. and *Pseudomonas* spp. However, the number of OTUs belonging to these two genera was relatively small, thus excluding them as the main candidate bacterial pathogens. In samples from subjects clinically diagnosed with NS and who later received a definite diagnosis, after excluding contaminating bacteria associated with a small number of OTUs, *A. kestanbolensis*, *G. vulcani*, *K. oxytoca*, and *A. guillouiae* were found to co-exist, suggesting coinfections maybe with multiple bacterial species in NS. We observed that *Anoxybacillus kestanbolensis* was associated with the largest number of OTUs in the 20 clinically confirmed NS samples, followed by *G. vulcani*; both species are gram-positive bacilli. *Klebsiella oxytoca* and *A. guillouiae*, associated with a slightly smaller number of OTUs, are gram-negative bacilli. These bacteria could not be cultivated using traditional bacterial culture methods; therefore, to verify the NGS results, we performed PCR amplification using specific primers followed by Sanger sequencing. These results of NGS were consistent with these findings.

The primary drugs used to treat patients with NGS-positive NS in the present study were latamoxef, ceftazidime, ceftriaxone, and penicillin (see Additional file 2), and subjects were hospitalized for an average of 5 days prior to discharge following symptom improvement, which was shorter than the 12.3 day treatment time reported by Wagstaff et al. for EOS patients [23], and the patient's prognosis was good, indicating a high cure rate for NS caused by these four bacteria. In contrast, 25 unconfirmed cases of NS were empirically treated with third-generation cephalosporin antibiotics and discharged after 3–7 days with symptom improvement. Latamoxef is a member of the oxacephem family and has an antibacterial spectrum similar to third-generation cephalosporin antibiotics (ceftazidime and ceftriaxone). These drugs can treat single or multiple pathogenic infections, with antibacterial activity against both gram-positive and gram-negative pathogens. Penicillin is efficacious in the treatment of infection-induced bacteraemia and sepsis.

The underlying diseases for NS in this study were primarily neonatal infectious pneumonia, neonatal hyperbilirubinemia, and neonatal meconium aspiration syndrome, all of which are common in newborns. Some patients with NS presented with double infections or multiple cross-infections. Although patients presented with different types of underlying diseases, four main bacteria, namely, *A. kestanbolensis*, *G. vulcani*, *K. oxytoca*, and *A. guillouiae*, were commonly identified. These bacteria were also identified in our previous study on male and female

urogenital diseases and are commonly present in the genitourinary tracts of men and women [5]. Thus, these bacteria can enter the foetal blood circulation through the foetal skin, oral cavity, or respiratory mucosa during intrauterine infection or delivery owing to the compression of the birth canal or other reasons. These four kinds of bacteria may temporarily enter the blood; however, with no way to isolate and grow these bacteria, further study is required to determine whether they cause NS.

Radchenkova et al. [24] isolated *A. kestanbolensis* from Bulgarian hot springs. Dulger et al. [25] isolated *A. kestanbolensis* sp. nov. from the mud and water samples of Turkish hot springs, but this bacterium has not been isolated in humans, nor has it been reported as related to human diseases. Sürmeli et al. [26] reported that α-L-arabinofuranosidase subjected to directed evolution and cultivation using *G. vulcani* GS90 exhibits improved activity, such as higher thermal stability but lower alkaline stability. Reportedly, the above-mentioned two gram-positive bacilli show high-temperature resistance, are difficult to isolate and culture at 37 °C, and have high nutritional requirements. Few reports are available on *G. vulcani*. Nazina et al. [27] isolated a new type of thermophilic spore-forming strain, GaT, from hot springs in northern Russia and found that this strain is genetically and phenotypically different from the known *Geobacillus* strain. Although *K. oxytoca* and *A. guillouiae* in the present China-based study belong to the same genera as *K. pneumoniae* and *A. baumannii* reported in other countries, some interspecies differences do exist.

Conclusions

Most reported bacterial pathogens in NS are resident bacteria or conditional pathogenic bacteria on the skin surface. To confirm that these are genuine bacterial pathogens, it is necessary to collect multiple blood samples from multiple skin sites to exclude contaminating bacteria and successfully culture the same bacteria in all samples. *Staphylococcus* spp. and *E. coli*, which have been frequently reported in clinical studies, were not detected in our samples. Thus, the bacteria detected in other clinical samples could contaminate bacteria from the skin surface due to improper skin disinfection or blood collection. In future research, the isolation, culturing, and identification of pathogenic bacteria will be conducted to further understand their biological properties and pathogenic mechanisms. NGS technology has high application value in the detection of pathogens in neonatal sepsis. It can screen rare and difficult to cultivate pathogens without the need for specific amplification primers. However, there are also certain shortcomings, such as NGS false-negative results caused by extremely low pathogen content or DNA degradation in the blood and the inability to know the antibiotic resistance of pathogens.

Abbreviations

Bp	Base pair
EOS	Early-onset sepsis
ITS1	Internal transcribed spacer 1
LOS	Late-onset sepsis
NGS	Next-generation sequencing
NS	Neonatal sepsis
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
UPGMA	Unweighted pair group method with arithmetic mean

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43054-024-00284-0.

Supplementary Material 1.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation and the first draft of the manuscript was written by YW. Data collection was performed by WMG. Data analysis was performed by ZNW. Specimen collection and testing were performed by MJL. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and this study was approved by the Shenzhen Seventh People's Hospital Ethics Committee (No. 20170204), and informed consent was obtained from the guardians of all subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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