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Characterization of a Highly Virulent *Klebsiella michiganensis* Strain Isolated from a Preterm Infant with Sepsis

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Purpose: *Klebsiella michiganensis* is an opportunistic pathogen that causes an increasing number of serious infections. This study aimed to investigate the etiology of the severe clinical symptoms of sepsis in preterm infants and the characterization of *K. michiganensis* isolates.

Patients and Methods: Whole-genome sequencing (WGS) was performed on three strains isolated from an infected preterm infant. Additionally, the genomic sequences of 534 *K. michiganensis* strains were obtained from the NCBI database. To gain deeper insights into these strains, we utilized the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), and Pathogen Host Interactions (PHI) database annotation tools for comprehensive gene function analyses. Moreover, the multilocus sequence typing (MLST), EasyCGtree, and virulence factor database (VFDB) were employed to determine the sequence types (STs), construct phylogenetic trees, and identify potential virulence factors.

Results: Sequence analysis found that the three isolated strains had identical sequence characteristics and did not correspond to any of the known ST types. Virulence factor analysis revealed that the three strains harbored *mrkABCDFHIJ, fimABCDEFGHIK, entABCDEFS, fepABCD*, and capsule genes. These virulence factors are likely to play crucial roles in enhancing adhesion and metabolic capabilities, resisting phagocytosis (inducing immune cell damage), and ultimately contributing to prolonged bacteremia. The phylogenetic tree and comparative genomics of virulence factors showed the genetic and virulence factor diversity of the currently reported *K. michiganensis* strains.

Conclusion: We identified a novel strain of *K. michiganensis* that exhibits high virulence and leads to severe septicemia phenotypes in preterm infants. Furthermore, comparative genomic analysis of previously reported *K. michiganensis* strains revealed the existence of three clades. This comprehensive analysis provides novel insights into the genetic relationships and virulence factor profiles of diverse strains of *K. michiganensis*. In future, it will be necessary to investigate the concept of the high virulence of *K. michiganensis* to determine the treatment method.

Keywords: *Klebsiella michiganensis*, whole-genome sequencing, preterm infant, sepsis

Introduction

The *Klebsiella oxytoca* complex is a member of the normal gut flora with a colonization rate of approximately 4.6% in preterm infants.[1] However, the *K. oxytoca* complex is an important human pathogen that can cause a variety of infections including bacteremia, pneumonia, and antibiotic-associated hemorrhagic colitis.[2] Research suggests that neonatal necrotizing enterocolitis may be caused by the growth of this opportunistic pathogen.[3,4] In addition, as many previous

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diagnostic results incorrectly classified *K. oxytoca* complex as *K. pneumoniae*, its contribution to clinical infections may have been underestimated.

Members of the *K. oxytoca* complex are highly diverse and include *K. michiganensis, K. oxytoca, K. pasteurii, K. spallanzanii*, and other new species.[5] *K. michiganensis* was seldom reported in hospitals in the past but has been gradually increasing in recent years,[6–8] with high homology and similarities in the molecular characteristics of *K. michiganensis* and *K. oxytoca*. However, few studies have reported the genomic characteristics of *K. michiganensis* strains isolated from clinical patients, especially their virulence profiles.

Routine clinical etiological identification, such as pathogen culture, 16S rRNA sequencing, and polymerase chain reaction, could not identify specific isolate strains. The use of whole-genome sequencing (WGS) in taxonomic, clinical, and epidemiological studies has led to a greater understanding of the genetic diversity of *K. michiganensis*. In this study, we aimed to investigate the etiology of the severe clinical symptoms of sepsis in a preterm infant and the characterization of *K. michiganensis* isolates recovered from multiple positive bacterial cultures. For this purpose, we collected samples from a preterm infant with severe sepsis at Capital Institute of Pediatrics for WGS. In addition, the genomic sequences of 534 *K. michiganensis* strains were obtained from National Center for Biotechnology Information (NCBI) for phylogenetic and virulence factor analysis. Virulence factor analysis explained the severe manifestations of clinical sepsis and identification of this pathogen in the preterm infant presented new *K. michiganensis* strains for better understanding the pathogenesis of sepsis.

Materials and Methods

Bacterial Isolation, Identification, and Antimicrobial Susceptibility Testing

Ko6508, Ko6509, and Ko6562 strains were isolated from blood specimens of a preterm infant with sepsis at the Children's Hospital, Capital Institute of Pediatrics. One milliliter of peripheral venous blood was collected and inoculated into a blood culture bottle. Once the incubator signaled an alert, an appropriate amount of culture medium was transferred onto Columbia blood agar plates and chocolate blood agar plate medium. These plates were then incubated at 37°C in a 5–10% CO_2 incubator for 20–24 hours. After incubation, individual colonies were isolated, and strain identification was performed using the VITEK® 2 Compact and mass spectrometry (VITEK/MS). Ko6508, Ko6509, and Ko6562 were initially identified as *K. oxytoca* by VITEK/MS and finally corrected as *K. michiganensis* by average nucleotide identity (ANI) analysis using Pyani-0.2.7. Results of the antimicrobial susceptibility test were interpreted according to CLSI guidelines.[9] The strains were preserved in glycerol cryopreservation solution and stored at −80°C.

Genome Sequencing, Assembly, and Annotation

Genomic DNA was extracted from each strain using the traditional Sodium Dodecyl Sulfate (SDS) DNA extraction method.[10] The DNA samples were randomly fragmented by the Covaris ultrasonic crusher to a size of 350 bp. After processing the DNA fragments, the NEBNext®Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) was used to prepare the entire library through end-polished, A-tailed ligation with the full-length adaptor, purification, PCR amplification, and other steps. The whole genomes of the three strains were sequenced using Illumina NovaSeq PE150 at Beijing Novogene Bioinformatics Technology Co., Ltd. After pre-processing, clean data were obtained, which were assembled using SOAP denovo (version 2.04)[11,12] and finally integrated using CISA software.[13] The genome size was estimated using K-mer statistical analysis before assembly. Gapclose (version 1.12), and other software packages were used to optimize the preliminary assembly results and fill holes to obtain the final assembly results. Fragments smaller than 500 bp were filtered out, contaminated samples were decontaminated again, and statistical analysis and subsequent genetic prediction were performed. Reads were compared to the assembled genome sequence, and the GC content and coverage depth of the reads were calculated to summarize the GC bias and genome repeats.

Genome Component Prediction and Gene Function

GeneMarkS (version 4.17) software (http://topaz.gatech.edu/GeneMark/) was used to retrieve the related coding gene.[14] Scattered repeat sequence prediction was performed using RepeatMasker (version open-4.0.5) software.[15] Tandem repeats

were analyzed using Tandem Repeats Finder (TRF; version 4.07b).[16] tRNA was predicted using tRNAscan-SE (version 1.3.1) software, and rRNA was predicted using rRNAmmer (version 1.2) software.[17,18] The Rfam database was first annotated for comparison, followed by the cmsearch program (version 1.1rc4) (parameter default) to determine the final sRNA.[19,20] Based on the sequence composition, IslandPath-DIOMB (version 0.2) software was used to predict gene islands,[21] which can be detected by phylogenetic bias and mobility genes in phylogenic sequences, such as transposase or integrase, to identify gene islands and potential horizontal gene transfer. Prophages in the sample genome were predicted using phiSpy (version 2.3) software, and CRISPR identification was predicted using CRISPRFinder (version 1.0).[22,23]

Diamond comparison (E-value ≤ 1e-5) was performed between the protein sequences of the predicted genes and the functional databases. For each sequence comparison result, the comparison results with the highest score (default identity ≥ 40%, coverage ≥ 40%) were selected. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), Non-redundant Protein Database (NR), Transporter Classification Database (TCDB), and Swiss-Prot databases were used to annotate the genes of the strains. All potential virulence factors were analyzed online using a VFanalyzer on the virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/).[24] The SNPs were obtained by sequence analysis using Snippy (4.6.0).

Collinearity Analysis and Phylogenetic Analysis

In this experiment, collinear alignment of the *K. oxytoca* genome was performed using Mauve 2.3.1 multiple sequence alignment software, and the program parameters were set to default values.[25] The publicly available genomes of *K. michiganensis* isolates (n = 567, updated April 25, 2024) were downloaded using RefSeq from the NCBI. ANI analysis of the 567 isolates was performed against the reference strains of *K. michiganensis* THO-011. A total of 539 publicly available *K. michiganensis* strains were mapped to the *K. michiganensis* reference strain. The genomic sequences of the three strains were input in the FASTA format for phylogenetic analysis and inference of their evolution. A phylogenetic tree of the 537 strains (534 publicly available strains and Ko6508, Ko6509, and Ko6562) of *K. michiganensis* was constructed using EasyCGtree.[26] Visualization of the phylogenetic tree analysis was performed using iTOL v6 (https://itol.embl.de/).

Results

Case and Microbial Detection Results

The preterm infant was born at a gestational age of 31^{+4} weeks and weighed 1500 g. Two hours after birth, the patient was admitted to our hospital with respiratory difficulties. Upon admission to hospital, the infant was dull in activity with mild pallor. Infection screening revealed a high white blood cell (WBC) count (Figure 1A). A provisional diagnosis was NRDS and neonatal infection. After 41 days of treatment with multiple antibiotics in the hospital, the patient recovered and was discharged. One *K. michiganensis* strain was isolated from the blood specimens on day 11 of hospitalization. Two other *K. michiganensis* strains were subsequently isolated on days 15 and 16 of hospitalization. The three strains

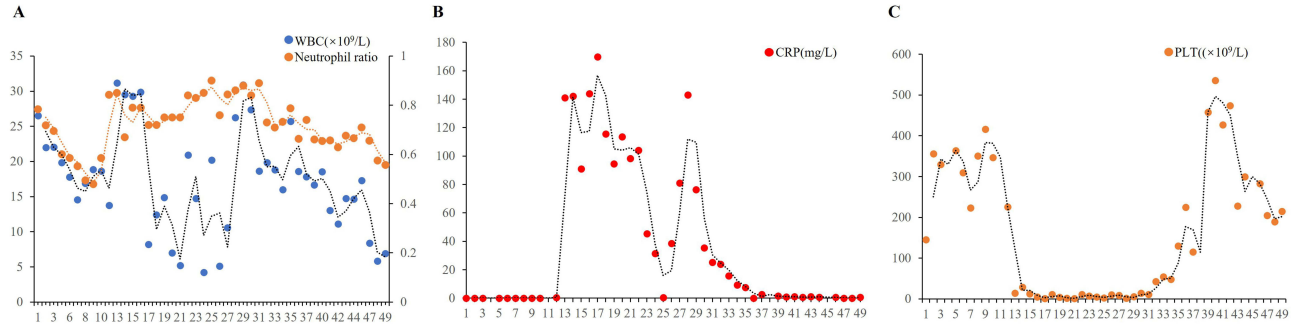

Figure 1 Complete blood cell count. (**A**) Axis of abscissa represents days of hospital stay; main ordinate (left) is white blood cell (WBC $\times 10^9$/L) count and secondary ordinate (right) is the neutrophil ratio. Blue dots represent WBC counts and black dashed lines represent count trends. Orange dot shows the neutrophil ratio and orange dotted line represents the trend. (**B**) Red dot indicates the level of C-reactive protein (CRP, mg/L). (**C**) Orange dot indicates the platelet (PLT, $\times 10^9$/L) count.

were initially identified as *K. oxytoca* by VITEK/MS, and high-throughput sequencing of pathogenic microorganisms revealed high-confidence confirmation and relative abundance of 4.73%. However, the three strains were mapped to *K. michiganensis* by ANI analysis. Antimicrobial susceptibility testing revealed that the three strains were susceptible to sulfamethoxazole, piperacillin /tazobactam, tigecycline, cefoperazone/sulbactam, ticarcillin/clavulanate potassium, aztreonam, ceftazidime, ciprofloxacin, cefepime, doxycycline, amikacin, imipenem, levofloxacin, meropenem, minocycline, and tobramycin (Supplementary Table 1). However, clinical outcomes following treatment with these antibiotics were suboptimal, manifesting as persistent and severe septicemia in the affected preterm infant. Laboratory findings revealed increased C-reactive protein (CRP) levels and thrombocytopenia (Figure 1B and C).

Genome Features and Gene Function of Ko6508, Ko6509, and Ko6562 Strains

Given that the sequences of the three isolated strains were nearly identical and minimal in SNPs (Supplementary Table 2), Ko6508 was selected as a representative strain for subsequent analysis. The total length of the genome was 6.51 Mb. The genome sequencing circle diagram is represented by 44 contigs (Figure 2). The maximum cluster was 842,578 bp in length, minimum continuous length was 716 bp, coding region size was 5.96 Mb, and average GC content was 55.63%, respectively. There were 78 tRNA, 9 rRNA, 43 sRNA, and 5737 coding genes with an average length of 906 bp. Basic information on the whole-genome sequence of Ko6508 is presented in Table 1. The predicted genes in the Ko6508 genome were analyzed using the GO database for gene functional annotation and the KEGG database for pathway annotation (Figure 3A and B). KEGG analysis revealed that these genes were enriched in environmental information processing and metabolism. The predicted genes in the Ko6508 genome were also translated into amino acids for COG functional classification (Figure 3C). Among the 5737 predicted genes in the genome of strain Ko6508,

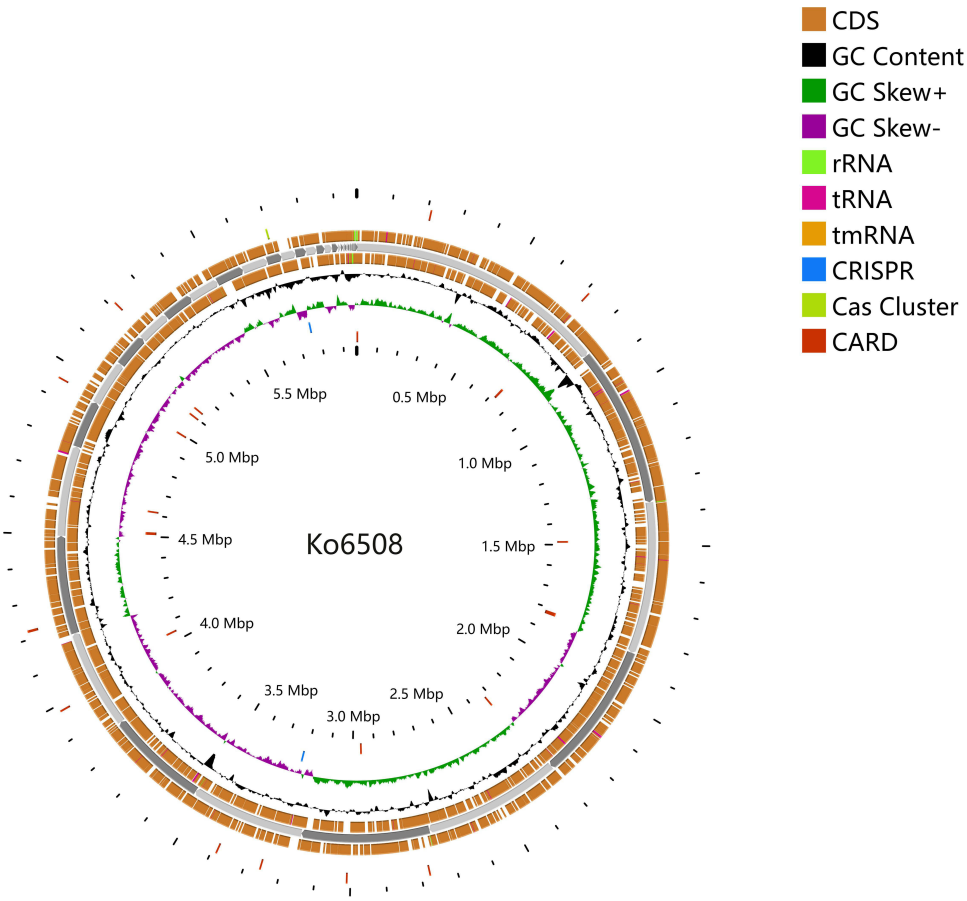

Figure 2 Circular map of Ko6508 chromosome. From inside to outside, first circle represents the scale, second circle represents GC skew, third circle represents GC content, and outer circle represents the position of CDS, tRNA, and rRNA on the genome.

Table 1 Genomic Characteristics of Ko6508

Strain	Ko6508
Size (Mb)	6.51
GC content of genome (%)	55.63
Contig number (>500 bp)	44
Maximum/minimum contig length (bp)	842,578/716
Contig N50 (bp)	413596
Scaffold number	44
Gene number	5737
Coding region size (bp)	5963844
Gene average length (bp)	906
Coding region/genome length (%)	87.19
Ratio of intergenic region (%)	12.81
tRNA	78
rRNA	9
sRNA	43
Prophage	10
CRISPR number	3
Gene island	14
LTR	45
DNA	22
LINE	14
SINE	18
TR	135
Minisatellite DNA	103
Microsatellite DNA	3

5343 were compared with COG numbers, accounting for 93.13% of the total gene number. The functional classification results of the COG showed 24 classifications. Expression of genes related to carbohydrate transport metabolism, amino acid transport metabolism, and transcription was high. The PHI phenotype classification revealed 429 reduced virulence genes (Figure 3D). However, the increased virulence (hypervirulence) of Ko6508 was 40 genes, and the unaffected pathogenicity was 211 genes. These results suggested that Ko6508 has a high degree of pathogenicity and hypervirulence, which may damage the host. We used the contig sequence for comparison, and the genome collinear results were relatively low for Ko6508, *K. michiganensis* DSM 25444, K. oxytoca NCTC 13727, and K. pneumoniae NTUH-K2044 (Figure 4). Generally, there are many locally collinear blocks (LCBs). There were insertions and deletions between the genomes, and there were more genome rearrangement events such as inversion and translocation.

Phylogenetic Analysis of K. michiganensis Genomes

To further investigate the population characteristics of *K. michiganensis*, MLST analysis of these *K. michiganensis* strains was performed. The results showed that strains Ko6508, Ko6509, and Ko6562 did not belong to any of the currently known ST types. There were 481 isolates (89.57%) with 120 ST types, and ST29 (45/537, 8.38%) was the most common, followed by ST43 (20/537, 3.72%), ST27 (19/537, 3.54%), and ST50 (18/537, 3.35%). A total of 56 isolates (10.43%) could not be assigned to known ST types. Our phylogenetic tree analysis revealed that *K. michiganensis* can be classified into three distinct clades: clade 1, clade 2, and clade 3. strains Ko6508, Ko6509, and Ko6562 belonged to clade 3 (Figure 5). These results offer enhanced precision in the classification of *K. michiganensis* strains via WGS and provide a deeper understanding of the genetic diversity, nonclonal transmission patterns, and population structure of *K. michiganensis* strains.

Comparative Analysis of Virulence Genes

To further understand the virulence factors of Ko6508, Ko6509, and Ko6562, we selected 290 isolates from clade 3 for comparative analysis of virulence genes (Figure 6). The results showed that type III fimbriae (*mrkABCDFHKIJ*),

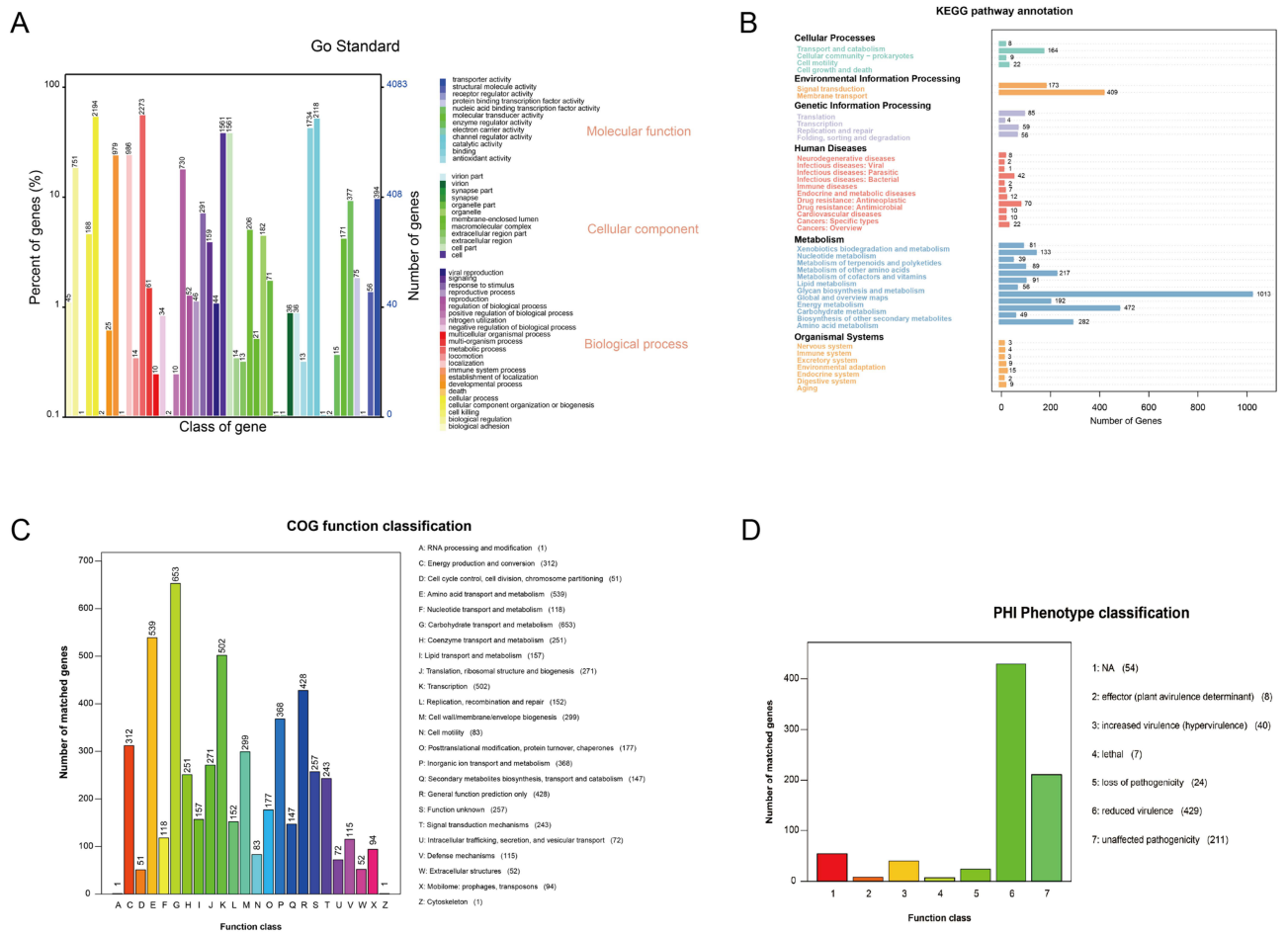

Figure 3 Genome features of Ko6508 genomes. (**A**) GO pathway annotation. (**B**) KEGG pathway annotation. (**C**) COG function classification of Ko6508. (**D**) PHI phenotype classification.

typeIfimbriae (*fimABCDEFGHIK*), ent siderophore (*entABCDEFS, fepABCDG, fes*), T6SSs, LPS rfb locus, capsule, and *acrAB*, and *rcsAB* genes were common in most *K. michiganensis* strains of clade 3. Closely related isolates had similar deletions in virulence genes. The results showed that evolutionarily structurally adjacent isolates possessed similar virulence phenotypes. Notably, *papC, PilW, iucCD, iroC and* toxin gene *clbQS* found in Ko6508, Ko6509, and Ko6562 were virtually absent in the other strains. In addition, the cell surface component *sugC*, magnesium transport gene *mgtB*, and *pla* were present in all three strains. These results suggest that Ko6508, Ko6509, and Ko6562 are highly virulent strains of *K. michiganensis*, leading to severe clinical manifestations in affected infants. Analysis of the drug resistance genes identified *acrA, acrB, acrD, mdtB, adeF*, and *oqxB* genes in the three strains.

Discussion

K. oxytoca and *K. michiganensis* appear to be the two main species of the *K. oxytoca* complex associated with human extraintestinal infections.[27] A multicenter study found that *K. michiganensis* was mainly distributed in Asian locations, specifically Pakistan,[28] while another study revealed that *K. michiganensis* strains were predominantly distributed in North America, Europe, East Asia, and Oceania, with 16 out of 32 strains originating from China.[7] In general, there are few studies on global context of *K. michiganensis*. Our analysis of the global background of 537 included isolates showed that the top five countries were the USA, UK, China, Switzerland, and Germany (Supplementary Figure 1). Further ST typing analysis revealed that the primary ST types were ST84 (11%) in the USA, ST88 (10%) in the UK, ST43 (10%) in China, ST85 and ST43 (both at 10%) in Switzerland, and ST98 (24%) in Germany (Supplementary Table 3). Before the pathogen is identified through the WGS, most clinical studies have not been subjected to precise

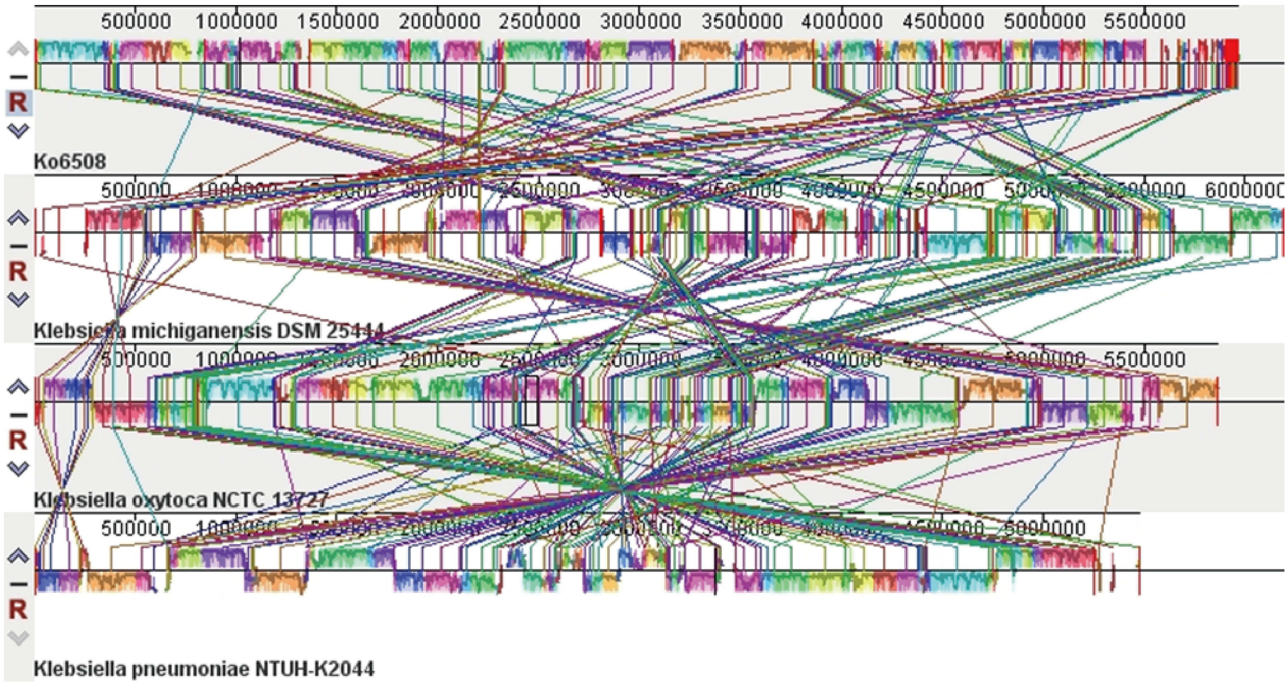

Figure 4 Global alignment of Ko6508 genomes. Modules of the same color connected by lines represent parts of the genome that are collinear, and there is no genome rearrangement inside. Completely white areas inside the block indicate that the genomes are not aligned and may contain specific components or mutations. Extraterritorial places indicate that no homology was detected between the genomes.

species identification, which can lead to incorrect estimates of pathogens. Here, the three studied strains (Ko6508, Ko6509, and Ko6562) isolated from blood specimens of a preterm infant, were initially identified as *K. oxytoca*, and finally as *K. michiganensis* by WGS.

The severe clinical symptoms of the patient may be related to the presence of virulence factors, such as fimbriae, capsules, efflux pumps, and siderophores. To further investigate the virulence factors of *K. michiganensis*, 537 *K. michiganensis* genomes were analyzed. Type III fimbriae (*mrkABCDFHIJ*) and typeIfimbriae (*fimABCDEFGHIK*) are common in *K. michiganensis* and contribute to bacterial adhesion, colonization, and phagocytic resistance. Capsules are required for virulence, and hypervirulent *K. pneumoniae* strains with gene mutations that affect capsule production show a strong reduction in virulence.[29] Therefore, the *capsule* genes identified in Ko6508 may facilitate immune escape and colonization. Genes related to iron uptake, such as salmochelin and aerobactin, are specific to high virulence.[30] They not only promote bacterial survival and capsule production but also affect virulence.[31] *entABCDEFS* and *fepABCD* have been identified in Ko6508, and these factors may play an important role in the colonization and invasion of pathogens. T6SSs are required to overcome microbiota-mediated colonization resistance and lead to successful host infection,[32] which was also observed in Ko6508. In addition, P fimbriae (*papC*) and type IV pili (*PilW*) may have contributed to strain adhesion and colonization. Iron uptake genes (*iucCD, iroC*) and colibactin genes (*clbQS*) facilitate survival and host damage.[30] All of these virulence factors were found in Ko6508 and were virtually absent in other *K. michiganensis* strains. Thus, we suggest that, akin to hypervirulent *K. pneumoniae*,[33] the concept of hypervirulent *K. michiganensis* should also be established.

However, virulence genes were commonly found in nearly all *K. michiganensis* strains, and the mere presence of those genes does not invariably correspond to a particular phenotype. While virulence genes certainly influence and dictate the structure and functionality of bacteria, it is worth noting that those genes are not fully expressed. Under certain circumstances, notably when the host's immune system is compromised, the opportunistic pathogen, such as *K. michiganensis*, has the potential to transition into a pathogenic state. At this point, *K. michiganensis*, which carries these various highly virulent factors, leads to long-term sepsis by increasing adhesion, resisting phagocytosis, increasing metabolic capacity, and inducing immune cell damage. Put simply, the phenotypic manifestation of bacteria is

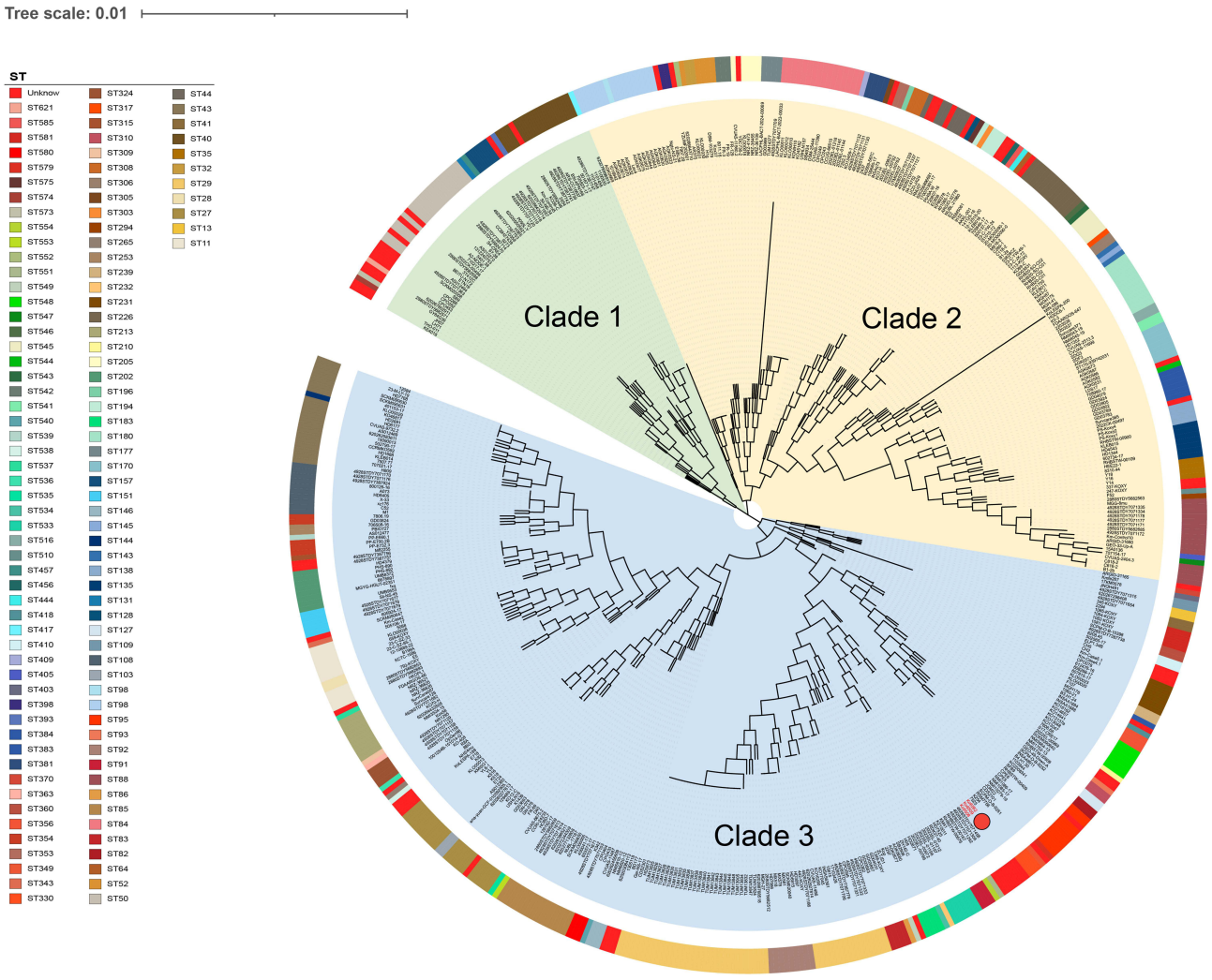

Figure 5 Phylogenetic analysis of 537 strains including Ko6508, Ko6509, and Ko6562. Phylogenetic tree generated using EasyCGTree. Red dot indicates position of Ko6508, Ko6509, and Ko6562.

a consequence of the interplay between genetic predisposition and environmental conditions. Thus, the identification of virulence genes may provide clues to understand the clinical significance and the regulation of those genes leading to long-term sepsis remains to be determined.

In this case, high inflammatory indicators, thrombocytopenia, and coagulation dysfunction were significant characteristics of newborns with Ko6508 sepsis. In severe infections, such as sepsis and septic shock, the coagulation and fibrinolysis systems are impaired. Disruption of the coagulation/anticoagulation balance can cause severe thrombosis or bleeding.[34] Notably, the protease (*Pla (Yersinia) pla* gene) was identified in Ko6508, which mediates coagulation dysfunction.[35] Another characteristic of newborns is thrombocytopenia. Studies have shown that sepsis can significantly alter the platelet transcriptome.[36] Accelerated platelet clearance and impaired platelet production are also responsible for reduced platelet counts. The effect of microbial pathogens on platelet function depends on the microbial strain. For example, *Escherichia coli* can directly induce platelet apoptosis by degrading the anti-apoptotic Bcl-xL protein.[37] Excessive thrombosis, especially sepsis-associated DIC, may deplete platelets.[38] In addition, high CRP levels boost antibody-mediated platelet destruction,[39] resulting in increased platelet clearance. In this case, the patient presented with prolonged sepsis, septic shock, and DIC symptoms, indicating various reasons for the decreased PLT count. Resistance–nodulation–division (RND) efflux pumps are important mediators of antibiotic resistance, and several RND efflux pumps have been identified, such as *AcrAB−TolC, MexAB−OprM*, and *AdeABC*.[40] In particular,

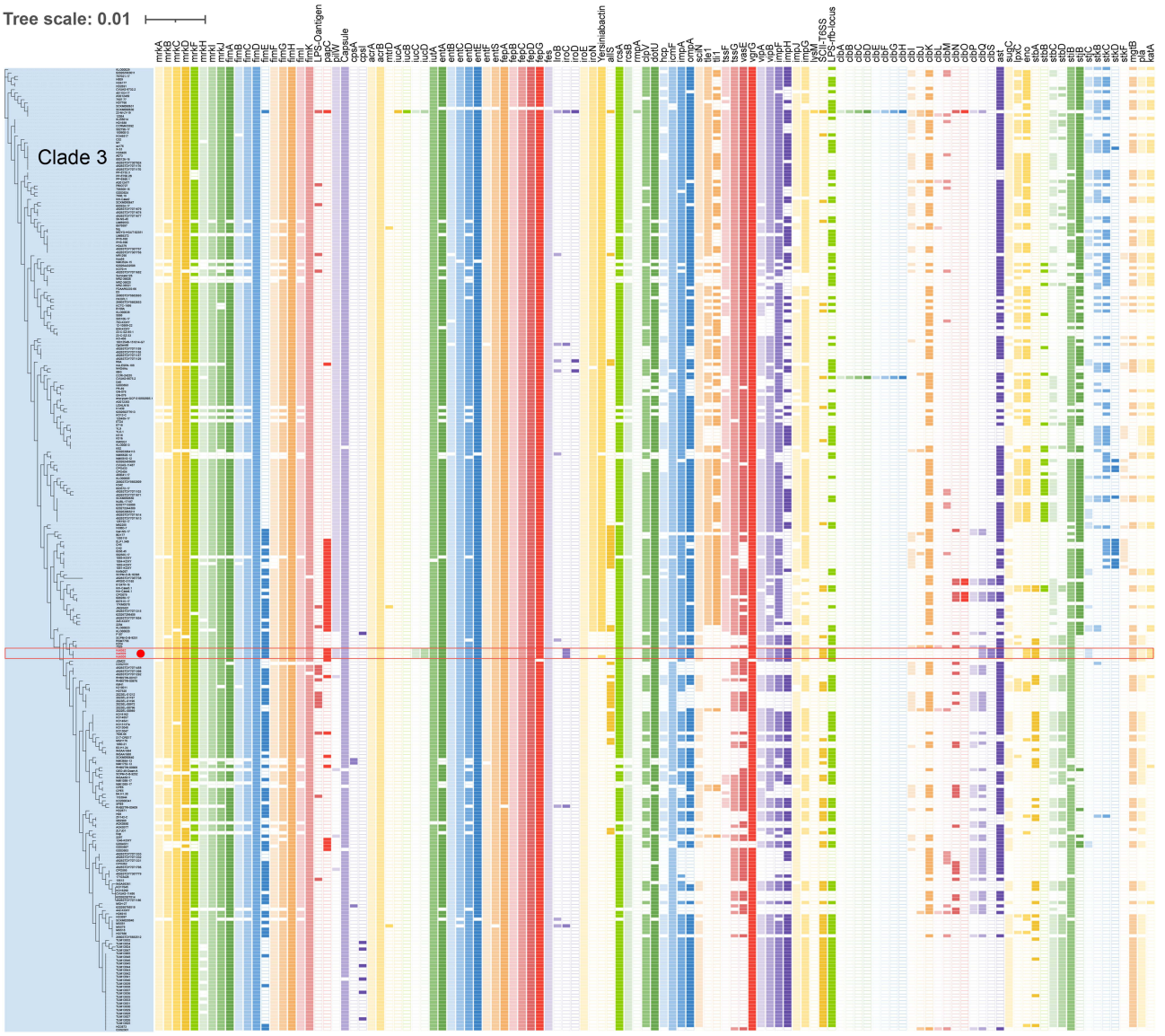

Figure 6 Heatmap of virulence genes in 290 strains including Ko6508, Ko6509, and Ko6562. Red dot indicates position of Ko6508, Ko6509, and Ko6562.

AcrAB−TolC has notorious substrate polyspecificity that confers resistance to nearly all classes of antibiotics, including carbapenems, tigecycline, and colistin.[41–43] However, the discovery of *acrAB* in Ko6508 did not explain why the antibiotics used in this case were ineffective. Because *acrAB* is prevalent in most strains of *K. michiganensis*, it is not unique to drug resistance or high virulence traits. In addition, drug-resistant genotypes and drug-resistant phenotypes were not completely consistent. For this newborn, clinical intervention guidance is still based on symptoms and drug sensitivity results.

In conclusion, WGS could precisely identify *K. michiganensis* and predict its virulence factors and resistance genes. For early-onset sepsis in newborns, we suggest that WGS should be used to identify the pathogenic bacteria more precisely. Although *K. michiganensis* is a conditional pathogen, it remains a deadly threat to immunocompromised individuals, such as premature infants, even in the case of drug-sensitive phenotypes. Virulence factors of *K. michiganensis* have conserved characteristics. However, there are obvious evolutionary differences between these strains, and many specific virulence factors remain to be identified. More importantly, rapid identification of highly virulent strains is of great significance for the management of *K. michiganensis*.

Data Sharing Statement

Whole-genome sequences have been deposited and are publicly available from the NCBI (Bioproject ID: PRJNA1110811). The datasets used in this study are listed in Supplementary Table 4.

Ethical Approval of Studies and Consent for Publication

This study was approved by the Ethics Committee of the Children's Hospital, Capital Institute of Pediatrics (SHERLLM2024002). This article relies solely on a retrospective analysis of case content without any violation of patient privacy. Written informed consents were obtained from the parents of the patient for publication of this study.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this article.

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