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Unraveling *Citrobacter koseri* Infection Outbreaks Using Next-generation Sequencing: A Clinical Case Study

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Abstract

Citrobacter koseri (C. koseri) is a normal gut microbe but can cause severe opportunistic infections in immunocompromised individuals and neonates. Due to sudden hospital outbreaks, it has recently gained attention. The lack of adequate environmental surveillance and poor understanding of the epidemiology of its spread warrant the use of more sensitive and superior analysis methods. Next-generation sequencing is a powerful and rapidly emerging tool yet to be validated for routine molecular diagnosis and epidemiology of *C. koseri* infections. In this study, the next generation sequencing (NGS) was used to investigate the outbreak of 4 cases of *C. koseri* bloodstream infection in a Neurology setting within a hospital over three days. The blood samples were tested for bacterial culture among the patients with fever (body temperature ≥38°C) and chills. Additionally, the environmental samples were also cultured. A total of 4 patients with chills and fever within three days of admission and cases of sepsis due to *C. koseri* were identified through routine diagnosis. *C. koseri* was also found in the sealing fluid of environmental samples. NGS analysis was conducted on four bacterial samples from the patient's blood. The NGS data showed that entire paired-end reads were assembled into a 4.5 Mb genome with an average GC content 54.66%. The phylogram is based on the global pan-genome, suggesting a distinct clade in the identified samples. Phylogenetic analysis of the 16S RNA showed two distinct clusters. Cluster 1 originated from CKB211, while Cluster 2 was isolated from patients in the same ward (CKB212, CKB213, and CKB214). The core-pan evolutionary analysis indicated that CKB211 had a distant evolutionary relationship with other strains and more subtle evolutionary relationships were also analyzed. This analysis is consistent with the physical distance of these patients. It strongly indicates a likely route of infection via shared saline, which was the common operational approach. The study provides unique insights into the rare infection caused by *C. koseri*, utilizing NGS and phylogenetic analysis. In the present study, although both 16S RNA and the core-pan phylogenetic tree can be used for the evolutionary analysis of *C. koseri*, the core-pan analysis involves a greater sequence and provides a more nuanced understanding of divergence. The study suggests using core-pan for

the evolutionary analysis of *C. koseri*. However, considering the limited sample size in this study, the applicability of this method remains to be explored.

Keywords

Citrobacter koseri, outbreak investigation, next-generation sequencing (NGS), 16S RNA, core-pan, Phylogenetic analysis

1. Introduction

C. koseri, formerly known as Enterobacter sakazakii, is a gram-negative, rod-shaped bacterium commonly found in the environment, including soil, water, and vegetation [1]. The entire genus of *Citrobacter* consists of facultatively anaerobic, motile, non-spore-forming bacteria that are oxidase-negative and utilize citrate as their sole carbon source. According to the DNA hybridization test, the genus *Citrobacter* comprises 11 species. Although *Citrobacter* is relatively less virulent, it occasionally causes human infection and is not commonly found in clinical specimens. However, it can be the source of several infections, such as those affecting the urinary tract, respiratory tract, abdominal cavity, skin and soft tissue, eye, bone, blood, and central nervous system infections [2].

C. koseri is a normal part of the intestinal flora in humans and animals. Still, it can cause opportunistic infections in immunocompromised individuals, particularly neonates, the elderly, and those with underlying medical conditions. In the immunocompromised population, infection with the bacteria can have serious consequences, leading to increased attention in recent years. The epidemiology of *C. koseri* infections is not well-defined due to the rarity of the infection. However, it has been reported to cause outbreaks in neonatal intensive care units, often associated with contaminated powdered infant formula. Infections in adults are generally associated with invasive medical procedures, such as catheterization, surgery, or mechanical ventilation.

C. koseri infections can manifest in various clinical presentations, ranging from meningitis, sepsis, urinary tract infections, and pneumonia. Symptoms may include fever, lethargy, poor feeding, vomiting, diarrhea, and respiratory distress. The most common clinical infections caused by *Citrobacter* species are from *Citrobacter freundii* (*C. freundii*) [3], followed by *C. koseri*. The most vulnerable organ of *C. koseri* infection is the urinary tract, followed by the respiratory tract [4]. Recently, *C. koseri* infections have mainly been as local infections in adults, such as postoperative local limb infection [5], infectious lens keratopathy [6], and bullous erysipelas of the leg [7]. Local infections can also occur in children. A case of a 6-week-old COVID-19-infected infant in France was reported of urinary tract infection with *C. koseri* [8], exhibiting mild symptoms. Besides, *C. koseri* could lead to serious consequences in individuals with weakened immune systems. The bacteria has been identified as the causative agent of liver abscesses in elderly patients[9] and can also specifically cause neonatal meningitis and brain abscesses, leading to intracranial pneumatosis and brain edema with poor prognosis [10]. There have been limited research methods for molecular typing of *C. koseri*. Next-generation sequencing (NGS) has emerged as an important method for studying *C. koseri* infections. NGS can identify changes in single genes between strains [11] and track the evolution of drug-resistant pathogens [12], making it a valuable tool in molecular epidemiology. NGS is suitable for outbreak investigation and molecular epidemiological surveillance [13], offering fast, accurate, and economical results.

This study investigated an outbreak of nosocomial infection caused by *C. koseri*, using NGS to trace its origin. It aims to provide effective prevention and control measures for nosocomial infection. Typically, diagnosing *C. koseri* infections involves laboratory testing, which includes isolating and identifying the bacterium from clinical samples, such as blood, cerebrospinal fluid, urine, or respiratory secretions. Recently, next-generation sequencing analysis has been employed to investigate *C. koseri* infection outbreaks, providing valuable insights into the organism's molecular epidemiology and the infection's transmission dynamics. Therefore, this study evaluates the diagnostic potentials regarding next-generation whole genome sequencing for epidemiological prospects and environmental surveillance of *C. koseri* infections.

2. Methods

2.1 Materials

From April 12 to April 14, 2021, four patients with chills and fever were successively identified in the neurology

setting. An investigation team comprising clinical, epidemiologic, and laboratory personnel was assembled. They visited the outbreak site to conduct investigations and environmental surveillance on the afternoon of April 14. The investigations involved reviewing patients who had developed fever and chills (body temperature ≥38°C) in the department from April 11 to 14. The blood samples from suspected cases were cultured using BacT Alert 3D (bioMerieux, France), and any resulting growth was identified by VitekII Compact (bioMerieux, France). Simultaneously, environmental specimens were collected from various sites, including air, room surfaces, infusion bag, infusion tubes, disinfectants, and the hands of medical staff, among others.

2.2 Next-generation sequencing

The Whole Genome Shotgun (WGS) strategy was used to construct libraries comprising various insert fragments. These libraries were paired-end (PE) sequenced using NGS methods to generate the Whole-Genome sequence based on the Illumina NovaSeq sequencing platform.

2.3 Genome Assembly and Annotation

Genome Assembly was performed using SPAdes (v3.15.3), following the instructions outlined in the tool's manual. The assembled sequences were retrieved, and the corresponding read was subsequently submitted to a public repository (identifier: BioSample: AMN26664682). Later, the genome assembly was visualized, along with annotations, using the online server Proksee (https://proksee.ca/). The annotations were plotted onto the circular genome map using the raw assembled files.

2.4 Alignment and Phylogenetic Analysis

Both 16s-level and core gene analyses were performed to achieve the species-level identification of the microbe from the sequence. Initially, complete genome hybridization was performed using the TYGS tool (https://tygs.dsmz.de/), and both hybridization scores and phylograms were considered for the analysis. Later, Muscle (version 3.8.425) aligned all full-length 16S RNA sequences and core-pan [14]. The default parameters of Muscle were used for the alignment. Following this, Gblock (version 0.91b) was applied to remove unreliable sequence alignment sections in the sequence alignment point [15]. The sequence was then converted into a format recognized by Mega7, and a phylogenetic tree was constructed using the Maximum Likelihood method in the Mega7 software package.

The nucleotide sequences have been submitted to GenBank under accession numbers SAMN26664682-5 (whole genome) and OM943553-6 (16S RNA).

2.5 Statistical Analysis

SPSS 20.0 was employed for the statistical analysis. Absolute numbers and proportions were used to describe the enumeration data, and the chi-square test was conducted to compare the differences in attack rates. A significant level of $P<0.05$ was considered statistically significant.

3. Results

3.1 Clinical case history and routine diagnostic outcome

C. koseri was isolated from the blood of 4 cases and confirmed for nosocomial infections. The cases experienced symptoms of cold, chills, and shortness of breath during the infusion process. No inflammatory reactions, such as redness, swelling, heat, and pain, were observed at the infusion or operation sites. Additionally, there were no symptoms of gastrointestinal infection, such as vomiting and diarrhea. The data on infection cases are presented in Table 1.

The onset times for the 4 cases were between April 12 to 14: one case occurred at 11:00 on April 12, two were concentrated at 10:00 on April 13, and one occurred at 10:35 on April 14. The median time from the onset of the cases to admission was nine days (5-15 days range), and the median time from the onset to the operation's completion was 3.5 days (3-4 days range). All cases developed during infusion. These four cases were distributed in 2 wards, patient CKB211 was in ward 403, and patients CKB212, CKB213, and CKB214 were in ward 405. Refer to Fig. 1 for further details.

Table 1. General information of 4 cases of infection

No	Sex	Age	Diagnoses	Endovascular devices	Maximum body temperature (℃)	Heating time	Site of infection	Other infections	Blood culture	surgery
CKB211	male	74	cerebral infarction	Superficial vein indwelling needle	38.5	4.12	Bloodstream infection	none	C. koseri	4.9 Cerebrovascular stenting
CKB212	male	69	cerebral infarction	Superficial vein indwelling needle	40	4.13	Bloodstream infection	none	C. koseri	4.9 Cerebrovascular stenting
CKB213	male	66	cerebral infarction	Superficial vein indwelling needle	38.5	4.13	Bloodstream infection	none	C. koseri	none
CKB214	male	65	cerebral infarction	Superficial vein indwelling needle	39	4.14	Bloodstream infection	none	C. koseri	none

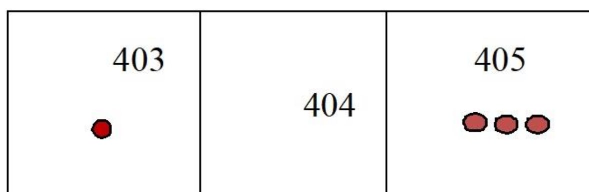

Figure 1. Spatial distribution of the 4 cases. Patient CKB211 was in ward 403, and patients CKB212, CKB213, and CKB214 were in ward 405.

All 4 cases were male, averaging 68.5 years (65-74 years old). Two cases underwent cerebral angiography on April 9 in preparation for stent implantation. They did not have infections of other parts from admission until the onset of the disease, and their body temperature was normal. Blood samples from infected cases were collected for bacterial culture during the period of temperature elevation when the patients experienced chills and fever, in which *C. koseri* was isolated. The susceptibility of these four bacteria to antibiotics was the same. The four patients recovered quickly after ceftazidime therapy.

Out of the 34 environmental samples, *C. koseri* was isolated from the indwelling needle eluate of patient CKB214 (numbered as CKS215). The bacteria were not detected in the rest of the samples. However, the bacterial colony count exceeded the standard limit in the samples collected from the hands of medical staff, bed sheets, and operating beds.

3.2 Outcome of Next-Generation Sequencing

The next-generation sequencing data on assembly revealed a highly significant and integrated genome map of *C. Koseri*. The genome assembly analysis demonstrated that entire paired-end reads were assembled into a 4.5 Mb genome, with a significant GC content in the initial 1 Mb and last 0.5 Mb of the genome. The GC skew representations (inner circles with green and violet graphs) represented relatively longer replication forks in the initial part of the genome spanning around 1 Mb region. In contrast, the later part of the genome, especially between the 4Mb to 4.5 Mb region, displayed a frequent change in GC skew across positive and negative values, indicating a more active and relatively increased number of replications forks. These findings were consistent with previously described maps and genome sequences of the *C. koseri*. Finally, the gene annotation was performed using the assembled sequence's post-analysis data, depicted over the circular genome map and as labels on the outermost circle. The intermediate circles represented open reading frames, repeats, and other variants of the genome (Fig. 2).

Furthermore, the Citrobacter pan-genome was analyzed to identify ~1,000 gene families across four experimental genomes. A cluster of Orthologous Group (COG) assignments was employed to determine all Citrobacter species' functional categories of core gene families. It was observed that these core gene families were unevenly distributed across various functional categories, such as transport and metabolism of carbohydrates (category G), translation, ribosomal structure, biogenesis, and inorganic ion transport and metabolism. Notably, most core gene differences were associated with transport and metabolism. Bacterial signal transduction systems are crucial in sensing environmental signals and adjusting cellular behavior and/or metabolism. The *C. koseri* strains identified in this study harbor a high pathogenicity island (HPI) cluster containing Yersiniabactin.

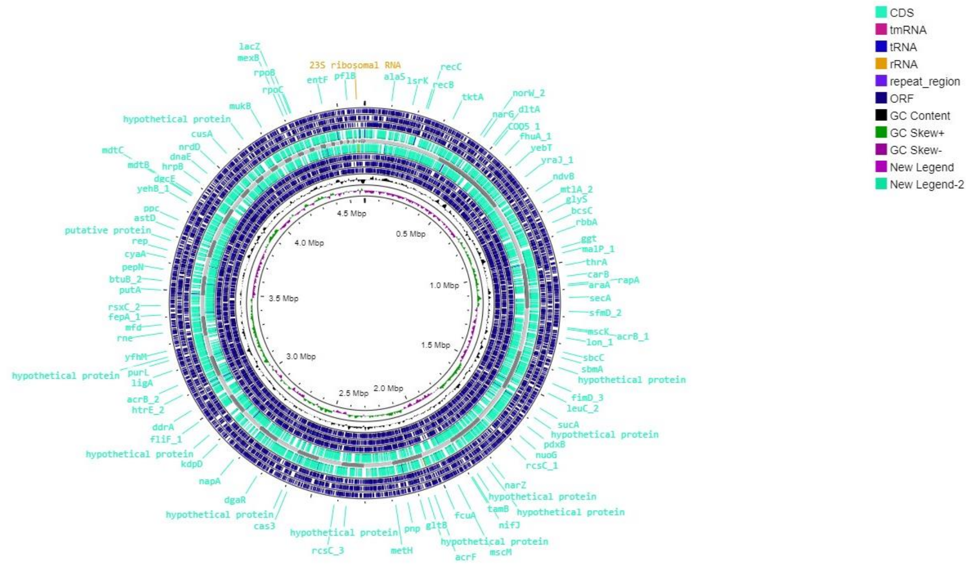

Figure 2. Annotated genome of C. Koseri showing the significant regions spread of 4.5 MbDNA sequence.

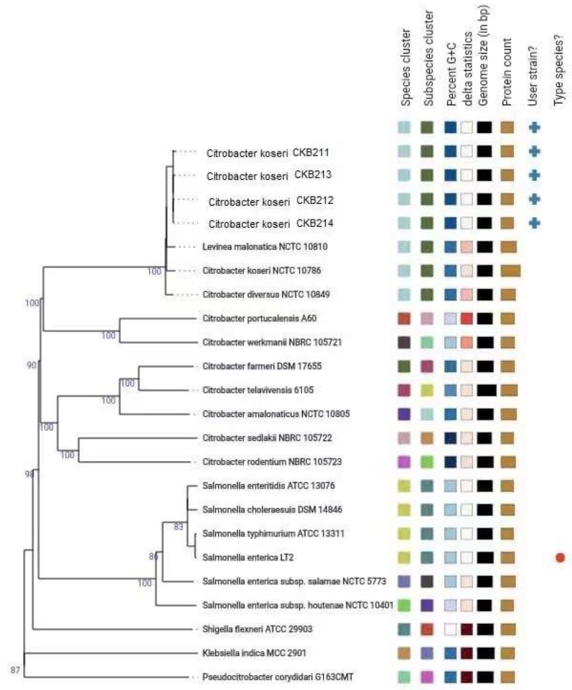

Figure 3. Phylogram based on global pan-genome and suggesting a distinct clade in the identified samples.

A genome-wide phylogram was also generated to identify the distinctiveness of the microbe and its genomic features, displaying a distinct cluster with prominent genomic similarity among the pan-genomes sequenced through paired-end reads using NGS (Fig. 3). Although the genome-based map was unable to differentiate the taxonomic variations across the experimental samples of C. Koseri, it was able to effectively discern and establish that C. Koseri samples were phylogenetically distinct and segregated on genome phylogram from other microbial species, particularly Salmonella spp. and other unrelated species of Citrobacter. This distinction was well-supported by a substantial bootstrap score, indicating the potential of NGS in molecular diagnostics and metagenomics. However,

it is important to note that due to redundancy and the existence of older taxonomic names in the database, *Citrobacter diversus* and *Levinea malonatica,* which are synonymous with *C. koseri,* are also represented within the same clad.

3.3 16S RNA-based phylogenetics

Whether or not there were differences across the samples isolated from blood remained unclear at the pan-genome level phylogeny. However, we were able to perform a 16S-based local phylogeny of selected sequences, resulting in a closely related phylogram that distinguished the sample types and hence supported the environmental surveillance efforts. Based on NGS, the 16S RNA full-length sequence analysis results are presented in Table 2. Notable differences in SNPs were observed between patient one and the other samples. A phylogenetic tree was also constructed, revealing that CKB212, CKB213, and CKB214 were slightly distant from CKB211 (Fig. 4).

Table 2. SNP sequence comparison of 16S RNA (The bold part is the difference sequence)

Sample	SNP sequence
CKB211	GGGAGGA**C**GGTGTT
CKB212	GGGAGGA**A**GGTGTT
CKB213	GGGAGGA**A**GGTGTT
CKB214	GGGAGGA**A**GGTGTT

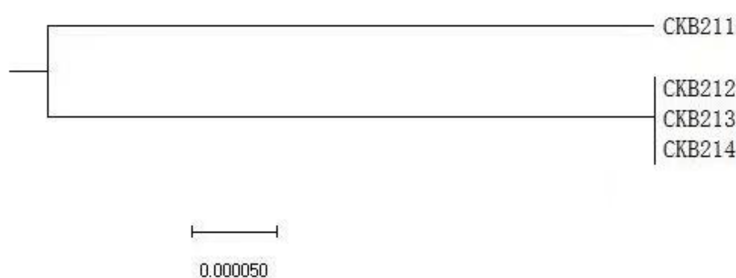

Figure 4. Phylogenetic tree analysis based on 16S RNA, CKB211 belong to a cluster, CKB212, CKB213, and CKB214 belong to the same cluster.

Core gene and phylogenetics

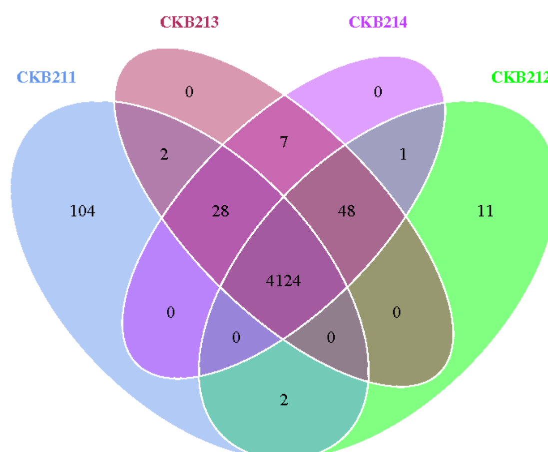

Figure 5. Venn diagram of the number of homologous genes between CKB211, CKB212, CKB213, and CKB214. The pan gene number is 4327, Core gene number is 4124, which covers 95.3%.

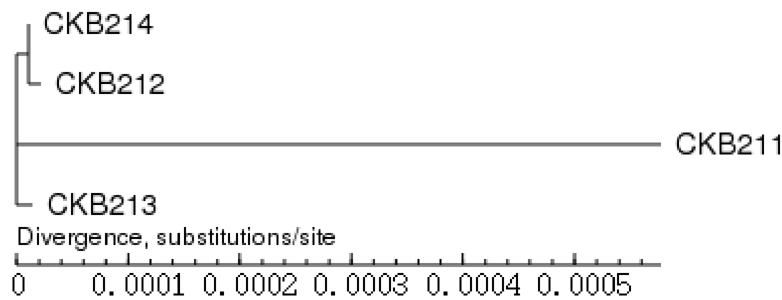

Figure 6. Display of phylogenetic tree based on the analysis of core genes. These four samples have a common intersection point, with CKB211 having a longer branch length and CKB212 and CKB214 having another intersection point.

As depicted in Fig. 5 and Fig. 6, these four samples share a common ancestor. CKB211 is evolutionarily more distant from the other three strains, which aligns with the physical distance between the patients. The patient of CKB211 was in one room, while CKB212 and CKB213 were in another room. Among these, the evolutionary relationship between CKB212 and CKB214 is closer.

4. Discussion

This study concluded that a nosocomial infection was caused by *C. koseri* infection by combining a comprehensive analysis of clinical manifestations, laboratory test results, and epidemiological investigations. For nosocomial bacterial and multidrug-resistant bacterial infections, transmission primarily occurs through contact, a finding supported by the analysis of nosocomial infection outbreaks in neonates in the global outbreak database. In these cases, contact transmission of nosocomial infection outbreaks accounted for 82%, while invasive procedures accounted for 12% [16]. According to a recently reported postoperative local infection, the patient had a history of *C. koseri* prostatitis two months before the operation [5], which might have resulted from self-infection.

Regarding the source of infection, initial consideration was given to contact transmission and invasive procedures. It was initially assumed that the venous indwelling needle and the sealing solution were contaminated through nursing operations, while surgical infection and drug contamination were excluded. This assumption was based on the following reasons: Firstly, the symptoms occurred more than three days after the operation, and there were no signs of inflammatory reaction, such as redness, swelling, heat, and pain at the wound site. Secondly, after one day, the first patient (Ward 403) began experiencing fever symptoms, and patients in Ward 405 exhibited symptoms afterward. Thirdly, the intravenous indwelling needle would be sealed with saline after the intravenous drip, which multiple individuals used. Lastly, other patients who received infusion simultaneously were not infected, and the bacterial culture of infusion liquid and drug samples were negative, ruling out infusion fluid and drug contamination as the cause.

In this study, 16S RNA revealed a common origin among CKB212, CKB213, and CKB214. 16S RNA sequences were highly conservative, making it difficult to distinguish between the different species of *C. koseri*. However, core-pan phylogenetics suggested that CKB211 is evolutionarily more distant from the other three strains, while the evolutionary relationship between CKB212 and CKB214 was closer. Therefore, core-pan analysis involves a larger sequence scope and provides more nuanced insights into divergence.

These patients were old and suffered from multiple diseases associated with compromised immunity. Simultaneously, the patients paid little attention to personal hygiene and did not take showers or maintain proper cleanliness during hospitalization. As a result, a variety of bacteria flourished in the surrounding environment. Furthermore, saline became contaminated due to non-standard nursing procedures, which resulted in the spread of pathogenic bacteria through intravenous injection operations.

The *C. koseri* strains in this study harbor a high pathogenicity island (HPI) cluster containing Yersiniabactin yet exhibit high drug sensitivity. The infection was successfully controlled after antibiotic treatment, resulting in a swift return of the patient's body temperature to normal. Studies have demonstrated that the resistance of Citrobacter bacteria evolves as they acquire resistance to nearly all antibiotics through plasmid-mediated and chromosomally encoded genes. According to a previous study, the resistance rates of carbapenems were reported to be 0-0.65% for *C. koseri* and 2-15% for *C. freundii*. Likewise, *C. koseri* strains were found to be more susceptible to antibiotics

than *C. freundii* [17]. The nosocomial infection incident was related to inadequate compliance with hand hygiene by medical staff, insufficient adherence to aseptic procedures, and inadequate cleaning and disinfection of the ward environment.

To mitigate future occurrences of nosocomial infection outbreaks, medical staff must strictly isolate patients suspected of hospital-acquired infection and adhere to the "one person, one bottle" for intravenous injection procedures. Rigorous hand hygiene protocols before and after nursing operations must also be implemented. Additionally, it is imperative to enhance collaboration among various departments involved in infection management, including medical staff, nursing staff, laboratories, and other departments. This concerted effort will enable the establishment of effective nosocomial infection prevention and control measures, ultimately reducing the occurrence of nosocomial infection outbreaks in the hospital.

The study was to identify the rare infection caused by *C. koseri*, a bacterium capable of causing severe infections in humans despite its relatively limited recognition. Using NGS offers highly accurate results, and phylogenetic analysis enhances our understanding of the genetic relationships between different strains of bacteria, aiding in identifying the infection source. This study employed 16S rRNA and core-pan phylogenetic trees for the evolutionary analysis of *C. koseri*. Due to its greater sequence inclusion and nuanced divergence, the core-pan analysis is recommended for such evolutionary analysis. However, the suitability of this method for studies with larger sample sizes remained to be explored, considering the current study's limitation in terms of sample size.

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