


# Genomic Sequencing of Clinical *Cupriavidus gilardii* Isolates Revealed Their Diverse Antimicrobial Resistance Mechanisms

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**Purpose:** *Cupriavidus gilardii* is an emerging multidrug-resistant pathogen found in many environments and few clinical samples. The clinical infectiousness, pathogenicity, and resistance mechanisms of *C. gilardii* are still unclear due to the lack of clinical and sequencing data. We need to obtain insight into the clinical characteristics, virulence, and resistance mechanisms of *C. gilardii*.

**Patients and Methods:** We isolated five *C. gilardii* isolates from hospitalized patients and carried out assay, culture and genome sequencing. We analyzed the genomic features of clinical *C. gilardii* isolates and took insight into their clinical characteristics, virulence, and resistance mechanisms.

**Results:** These isolates were resistant to meropenem, gentamicin, and other antimicrobials due to intrinsic resistance genes. Furthermore, the sequencing results revealed the widespread presence of the *MCR-5.1* gene in *C. gilardii*. The virulence magnitude of *C. gilardii* is closely correlated with the number of virulence factors they carry. Some *C. gilardii* strains can acquire resistance to levofloxacin through *gyrA* gene mutation during treatment. The diverse antimicrobial resistance mechanisms challenge the treatment of *C. gilardii* infections.

**Conclusion:** We present the genomic characteristics of clinically isolated *C. gilardii* to improve (i) our understanding of this pathogen and (ii) treatment options.

**Keywords:** *Cupriavidus gilardii*, clinical characteristics, antimicrobial resistance, *MCR-5.1*, *gyrA*

## Introduction

*Cupriavidus gilardii* is an aerobic, Gram-negative, non-glucose-fermenting bacterium belonging to the family Burkholderia of the phylum Betaproteobacteria, characterized by copper resistance, a rod shape, and motility.<sup>1–3</sup> *C. gilardii* is widespread in many environments, including water and heavy metal-contaminated plants and soil.<sup>4–6</sup> As microbial culture and identification technology advance, the increasingly successful detection of *C. gilardii* has gradually attracted clinical attention.<sup>7,8</sup> *C. gilardii* had been isolated and identified from a variety of clinical samples, including cerebrospinal fluid, bone marrow, wounds, and respiratory secretions.<sup>9</sup> There are currently no recommendations for antimicrobial use to treat *C. gilardii* infections.<sup>8</sup> Moreover, the high case fatality rate of *C. gilardii* infections may be related to the characteristics of acquired drug resistance.<sup>8,10</sup> Compared with other clinically well-known pathogens, we know very little about the genome, clinical characteristics, and therapy of *C. gilardii*. To date, only six clinical infection cases have been reported,<sup>10–15</sup> and 11 non-clinical *C. gilardii* strains have been sequenced. The genomic differences between the clinical and environmental isolates still remain to be explored.

*C. gilardii* was first thought to be an opportunistic pathogen,<sup>10–12</sup> but recent studies have shown that *C. gilardii* infection also exists in immunocompetent patients.<sup>13,14</sup> Predisposing factors for *C. gilardii* infection remain unclear. *C. gilardii* is intrinsically resistant to many antimicrobials and could acquire drug resistance with continued antimicrobial therapy.<sup>10,13</sup> The acquired drug resistance has been reported to inhibit the efficacy of antimicrobials and even lead to fatal consequences.<sup>10</sup> Therefore, it is critical to determine the clinical infection characteristics and drug resistance mechanisms of *C. gilardii* to reduce the case fatality rate.

We found four cases of *C. gilardii* infection in our daily work recently, and all four patients were seriously ill. In the present study, we cultured and isolated the microbes from the four patients and analyzed their clinical characteristics. Moreover, the microbes were identified and whole-genome sequencing was performed. Their virulence, and drug resistance were studied and compared with those of other environmental isolates.

## Materials and Methods

### Clinical Characteristics

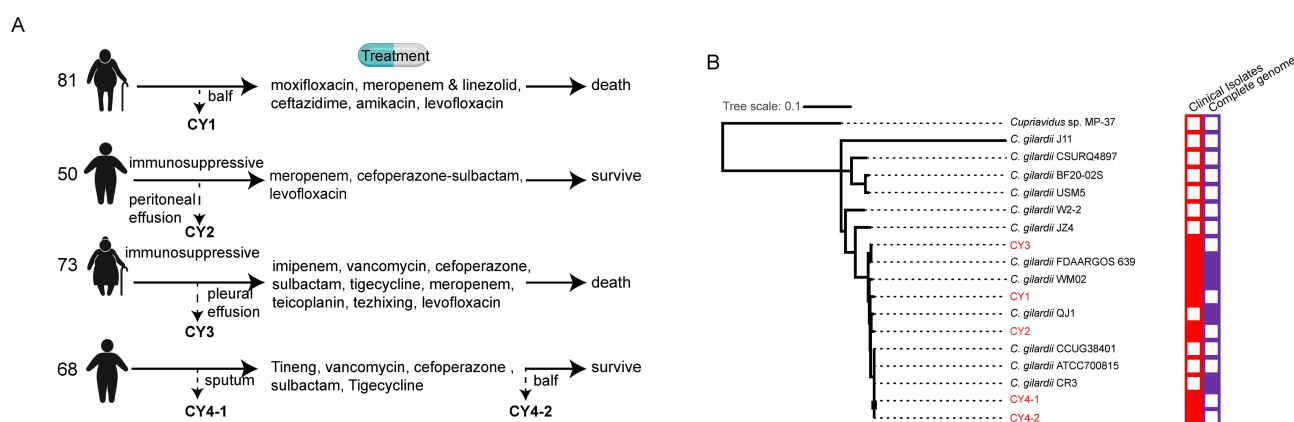
During the period from July 2020 to January 2021, four patients from different places in China were hospitalized in the Respiratory Intensive Care Unit (RICU) in Beijing Chao-Yang Hospital. Demographic and clinical data were obtained from electronic medical records, including personal history, comorbidities, symptoms, laboratory tests, length of stay in the RICU, severity scores (SOFA, APACHE II), outcomes, and follow-up.

### Isolation and Identification

From all four patients, we collected five body liquid samples (Figure 1A) and cultured the bacteria on blood agar plates under aerobic conditions for 24h at 37°C. Colonies were picked for identification using a VITEK MS Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (database version 3.2, bioMérieux Inc.). The minimum inhibitory concentration (MIC) assay was performed using broth microdilution methods per Clinical and Laboratory Standards Institute last guideline [CLSI M100Ed30].<sup>16</sup> The other antibiotics were tested using Disk Diffusion Assay (Kirby Bauer).

### Genome Extraction and Sequencing

Genomic DNA was extracted using Easy-Pure Bacteria Genome DNA Kit (TransGen Biotech) and quantified by a Qubit<sup>®</sup> 2.0 Fluorometer (Thermo Scientific). The whole genomes of the five *C. gilardii* isolates were sequenced using an Illumina NovaSeq PE150 platform at Beijing Novogene Bioinformatics Technology Co., Ltd., according to the library construction protocols of Illumina.



**Figure 1** Information on the clinical and genetic background of the five sequenced *C. gilardii* isolates. **(A)** Schematic diagram of the treatment process of the four patients. Dashed lines with arrows indicate where/when samples were collected and sample names. The used antimicrobials are listed in sequence. **(B)** Phylogenetic tree of the 16 *C. gilardii* strains based on the SNPs called using parSNP. Red and purple boxes indicate that the sample is clinically sourced and completely assembled, respectively. The strains sequenced in this study are shown in red font.

## Genome Assembly and Analyses

The raw sequencing reads were trimmed with Trimmomatic (v0.32) and then assembled using SPAdes (v3.10.0)<sup>17</sup> and SOAPdenovo.<sup>18</sup> The assembly results of the two software programs were integrated with CISA. We filtered the fragments below 500 bp. Annotation was performed using Prokka.<sup>19</sup> The annotated proteins were used to identify genes involved in antimicrobial resistance based on the CARD database (v3.2.2) using BLAST (v2.9). We only considered the genes with >70% coverage and  $\geq 70\%$  identity. A pathogen–host interactions database was used to analyze pathogenicity. Comparative genomic analysis was performed with a custom script that integrated BLAST (v2.9). The phylogenetic tree was generated using parSNP.<sup>20</sup>

## Results

### Clinical Characteristics

The clinical characteristics of the four patients (three males and one female) infected with *C. gilardii* are listed in [Table S1](#). Their age ranged from 50 to 84 years. Two of them were immunosuppressed (esophageal cancer), and the other two were not immunocompromised or immunosuppressed. Case reports are displayed as a flowchart in [Figure 1A](#). We used advanced antimicrobials such as cefoperazone-sulbactam, carbapenems, and vancomycin at the early stage for all four patients, but treatment was not effective. This may be correlated with the inherent resistance of *C. gilardii*. The treatment methods were adjusted, and of the four patients, two survived. Three patients got purulent infections, including empyema, mediastinal abscess, and lung abscess. The values of IgG/IgM, CD4<sup>+</sup>/8<sup>+</sup> T cells, and CD19<sup>+</sup> B cells were lower in the two patients who died than in the two patients who survived.

### Identification and Antimicrobial Susceptibility of *C. gilardii* Isolates

To characterize the pathogens, we cultured the pathogens from the body fluid (bronchoalveolar lavage fluid [BALF], peritoneal effusion, pleural effusion, and sputum) after the early treatment stage on blood agar plates. The MALDI-TOF MS assay results showed that the bacteria were *C. gilardii* (named strains CY1, CY2, CY3, and CY4-1). No other organisms were cultured.

Furthermore, we sampled the RICU environment (pool, ventilator, dining table, bed rails, bedside) during treatment, but all *C. gilardii* tests were negative, excluding the possibility of nosocomial transmission.

Interestingly, CY4-1 was initially sensitive to levofloxacin but acquired resistance during treatment. Thus, we re-cultured the drug-resistant bacterium from the sputum after treatment and named it *C. gilardii* strain CY4-2 ([Figure 1A](#)). For these five *C. gilardii* strains, the MIC was subsequently assayed using broth microdilution methodology ([Table 1](#)). The antimicrobial susceptibility profiles of *C. gilardii* strains showed a certain degree of resistance to 12 antimicrobials, including imipenem (IMI), ceftazidime (TAZ), cefepime (FEP), ceftazidime/avibactam (CZA), amikacin (AMI), meropenem (MERO), ceftolozane/tazobactam (C/T), aztreonam (AZT), piperacillin/tazobactam (P/T), tobramycin (TOB), colistin (COL) and ampicillin (AMP). All strains are resistant to Imipenem, different from environmental isolates but consist with some clinical isolates.<sup>10,12</sup> Both CY4-1 and CY4-2 showed resistance to colistin. CY4-2 was the only strain that was resistant to levofloxacin.

**Table 1** Antimicrobial Agent Susceptibilities

Strain	IMI	LEVO	TAZ	FEP	CZA	AMI	MERO	C/T	AZT	P/T	TOB	COL	AMP <sup>a</sup> (mm)
CY1	16	$\leq 0.5$	32	4	32/4	>32	>32	4/4	>16	>64/4	>8	$\leq 1$	6, R
CY2	8	$\leq 0.5$	32	4	32/4	>32	>32	8/4	>16	>64/4	>8	$\leq 1$	6, R
CY3	16	$\leq 0.5$	16	4	16/4	32	>32	>32/4	>16	>64/4	>8	$\leq 1$	6, R
CY4-1	8	$\leq 0.5$	32	4	32/4	>32	>32	8/4	>16	>64/4	>8	4	6, R
CY4-2	16	>4	>32	8	32/4	>32	>32	>32/4	>16	>64/4	>8	4	6, R

**Note:** <sup>a</sup>The test method is disc diffusion assay.

**Abbreviations:** IMI, imipenem; LEVO, levofloxacin; TAZ, ceftazidime; FEP, cefepime; CZA, ceftazidime/avibactam; AMI, amikacin; MERO, meropenem; C/T, ceftolozane/tazobactam; AZT, aztreonam; P/T, piperacillin/tazobactam; TOB, tobramycin; COL, colistin; AMP: Ampicillin.

## Genome Sequencing and Assembly of the Five *C. gilardii* Strains

We sequenced the genomes of the five *C. gilardii* strains using a PE150 NovaSeq system and analyzed the data. After assembly and annotation, we characterized the sequencing data and genome features (Table 2). The clean data sizes of all strains that could be used to assemble fragments were similar, yielding an average coverage depth of approximately 100× for each isolate. Due to the existence of repeat regions (especially the ribosomal RNA of *C. gilardii*) and the absence of long read sequencing, we could not assemble the complete genomes of the five *C. gilardii* strains with only NovaSeq sequencing data. However, this did not interfere with the analysis of the components and functions of the genomes. The number of coding sequences and GC content differed little between strains.

## Whole-Genome Phylogenetic Analysis

To analyze the genome similarity and the evolutionary relationships to other *C. gilardii* strains, we constructed a single-nucleotide polymorphism (SNP)-based phylogenetic tree using all reported *C. gilardii* genomes (12 in total) and *Cupriavidus* sp. MP-37 as outgroup species (Figure 1B). Most of the 12 reported genomes were drafted, and only four were complete genomes. Additionally, *C. gilardii* FDAARGOS 639 and WM02 were described to be a clinical isolate. All five sequenced strains were grouped into *C. gilardii*. CY4-1/2 showed a very high similarity (98%) to *C. gilardii* CR3, which was isolated from a tar pit. CY1, CY2, and CY3 were different from each other. The SNP-based phylogenetic tree showed that the infection sources are different. Since clinical case reports of *C. gilardii* infection are rare, attentions should be paid to the appearance of four cases in such a short period.

## Analysis of Antimicrobial Resistance Genes

*C. gilardii*, despite being an opportunistic pathogen, has intrinsic resistance to many antimicrobials. Clinical treatment of *C. gilardii* is challenged by the multidrug resistance. From the assembled genomes and annotations, we identified the antimicrobial resistance genes of the five clinical *C. gilardii* isolates (Table 3). Genes sharing at least 70% coverage and 70% identity to genes in the CARD antimicrobial resistance gene database were identified using BLASTP. Nine genes were found, including five genes encoding efflux pump systems (MuxC/B, MexB/D, and ceoB), three intrinsic resistance genes (OXA-837, AAC (3)-IVb, and ANT(3'')-Ib), and the colistin resistance gene MCR-5.1. Interestingly, the antimicrobial was changed during treatment. MCR-5.1 was found in many other *C. gilardii* strains present in a transposon. We compared the neighboring regions of the MCR-5.1 gene in *C. gilardii* strains (Figure 2). Six out of the 12 available *C. gilardii* genomes were found to possess MCR-5.1, of which WM02, QJ1 and CR3 were completely assembled. The adjacent regions of MCR-5.1 in CY4-2 are highly homologous to those of ATCC700815 and CCUG38401. Genes mdtH, chrB1, and hin always co-exist with the MCR-5.1 gene. Segments with this combination of genes are present in a variety of *E. coli* plasmids, such as pSGMCR103 and pEC1897-13, and on the *C. gilardii* QJ1 plasmid plas1. Furthermore, this segment was integrated twice into the QJ1 genome, adjacent and in opposite directions. Interestingly, we did not identify any apparent presence of plasmids in CY4-1 and CY4-2 based on the sequencing results. The integration site in CSURQ4897 is different from those in other strains, which is consistent with the phylogenetic tree in Figure 1. This suggests that the MCR-5.1 gene may be integrated into bacterial genomes via *E. coli*-like plasmids. The integration site in the *C. gilardii* genome varies depending on genetic differences.

**Table 2** Sequencing and Assembly Information of the Five *C. gilardii*

Strains	Raw Data(Mb)	Clean Data(Mb)	Mean Coverage	Total Contig (>500)	N50 Length(bp)	Size (bp)	GC Content (%)	No. of CDSs	No. of tRNAs
CY1	1814	1513	99	16	931,990	5,544,472	67.45	5120	53
CY2	1586	1359	102	17	654,248	5,437,752	67.52	4979	54
CY3	1588	1344	95	62	264,688	5,866,471	67.32	5540	55
CY4-1	1589	1358	94	65	527,680	5,918,194	67.31	5585	56
CY4-2	1488	1269	97	62	527,680	5,794,140	67.31	5460	53

**Table 3** Antimicrobial Genes of the Five Sequenced *C. Gilardii*

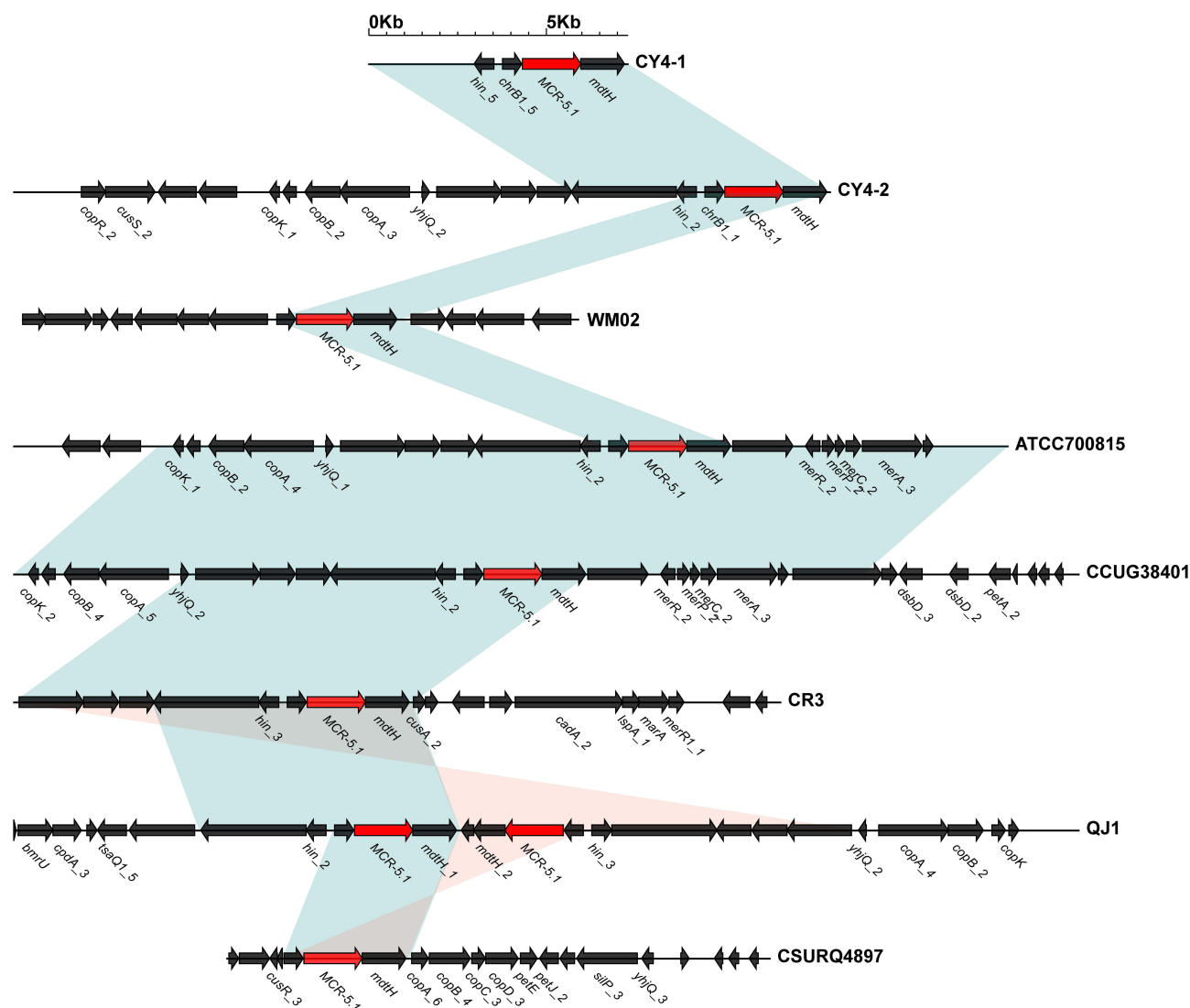
Genes <sup>a</sup>	Coverage/Identity(%)					Product	Resistance
	CY1	CY2	CY3	CY4-1	CY4-2		
<b>MuxC</b>	69.6/74.5	69.5/74.4	69.6/74.5	69.8/74.3	69.8/74.3	RND components in the <i>Pseudomonas aeruginosa</i> efflux pump system	Tetracycline, macrolide, aminocoumarin, monobactam
<b>MuxB</b>	97.0/79.1	97.0/79.2	97.0/79.1	97.0/79.1	97.0/79.1		
<b>MexB</b>	98.3/80.2	98.3/80.4	98.3/80.4	98.3/80.3	98.3/80.3	MexB is the inner membrane multidrug exporter of the efflux complex MexAB-OprM	Peptide, penam, diaminopyrimidine, sulfonamide, cephalosporin, macrolide, tetracycline, phenicol, fluoroquinolone, carbapenem, monobactam, aminocoumarin, cephamycin, penem
<b>OXA-837</b>	100/83.3	100/83.5	100/83.3	100/82.8	100/82.8	A class D OXA-like beta-lactamase	Penam, carbapenem, cephalosporin
<b>AAC(3)-IVb</b>	93.4/88.5	93.7/83.4	93.4/88.9	93.7/84.0	93.7/84.0	Novel aminoglycoside resistance gene	Gentamicin, tobramycin
<b>ANT(3'')-Ib</b>	94.0/85.5	-	82.4/87.7	-	-	Novel aminoglycoside 3''-adenyltransferase gene and aminoglycoside resistance gene	Spectinomycin, streptomycin
<b>ceoB</b>	97.6/81.3	97.6/81.2	97.7/81.1	97.6/81.1	97.6/81.1	Cytoplasmic membrane component of the CeoAB-OpcM efflux pump	Aminoglycoside, fluoroquinolone
<b>MCR-5.1</b>	-	-	-	100/100	100/100	Transposon-associated phosphoethanolamine transferase gene	Peptide-antibiotic, colistin
<b>MexD</b>	-	98.5/75.4	-	97.4/75.5	97.4/75.5	MexD is the multidrug inner membrane transporter of the MexCD-OprJ complex.	Aminocoumarin, tetracycline, phenicol, diaminopyrimidine, macrolide, cephalosporin, aminoglycoside, fluoroquinolone, penam

**Notes:** <sup>a</sup>The cutoff of the coverage and identity are 70% and 70%. Genes that under the cutoff were not shown.

According to the literature,<sup>21</sup> *gyrA* and *gyrB* are levofloxacin resistance-related genes. We compared the sequences of *gyrA* and *gyrB* from all *C. gilardii* strains (Figure 3A). The results demonstrated that there are mutations I83T and T514S in *gyrA* during the treatment, however, no mutation in *gyrB* were observed in *gyrB*, which may account for the reason for levofloxacin resistance (Table 3). This acquisition of resistance occurred during a 2-week treatment. The analysis of this longitudinal sample implied the emergence of acquired drug resistance of bacteria during the treatment, which may partially interrogate the high mortality rate of *C. gilardii* infection.

## Analysis of Virulence Factors

Virulence factors (VFs) in bacteria, including those involved in adhesion, invasion, toxin production, and immune evasion, are closely related to bacterial toxicity. The five strains isolated in this study showed different virulences, and two of them caused death (CY1 and CY3). We screened for known/potential VFs of 10 strains (five strains in this study and five closest strains in Figure 1A) from the latest virulence factor database<sup>22</sup> using VFAnalyzer.<sup>23</sup> The VFs numbers were counted according to different VF categories (Figure 3B). The total number of VFs ranged from 90 to 98. Both CY4-1 and CY3 had 7 more VFs than Cy4-2, mainly including biofilm formation and secretion system. Cy4-1 and Cy4-2 were isolated from the same patient, among which Cy4-2 was obtained after the patient recovered. Judging from the clinical data (Table S1), CY3 was also highly toxic. Interestingly, both CY3 and CY1, which caused the host patients died, have cell surface components. Though FDAARGOS 639 has cell surface components, there is no information to indicate its toxic in clinic. In general, the virulence magnitude of *C. gilardii* is closely correlated with the number of virulence factors they carry, with biofilm formation, secretion system, and cell surface components may play an important role.



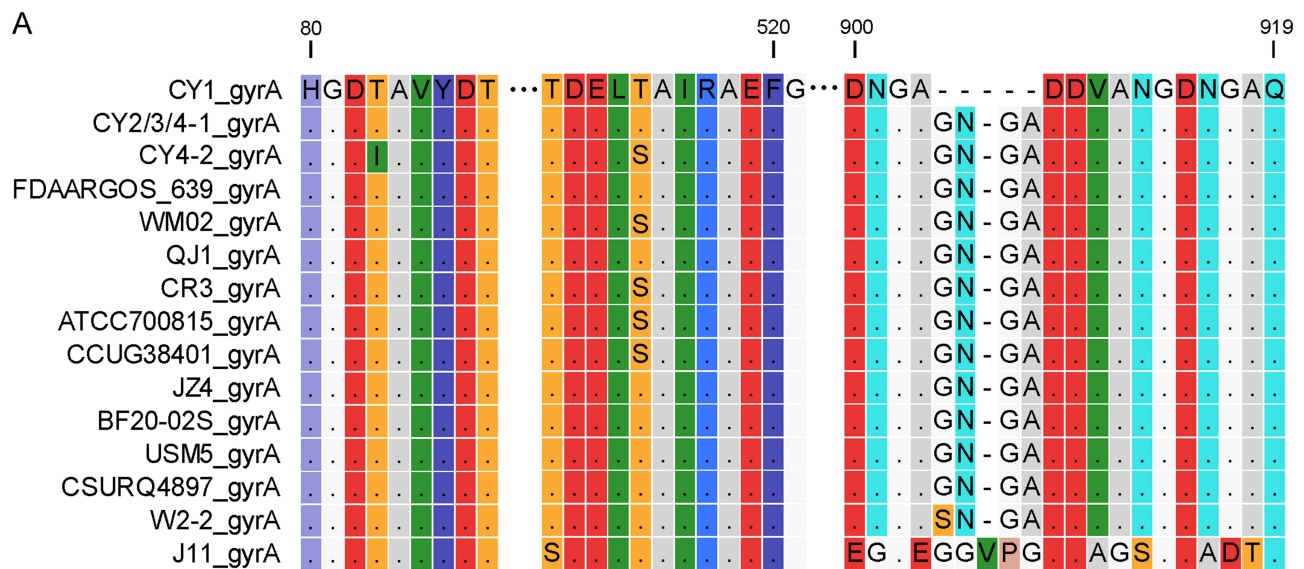
**Figure 2** Genomic regions near the MCR-5.1 gene. Homologies between different strains are linked by light green quadrilaterals. MCR-5.1 genes are highlighted in red.

## Discussion

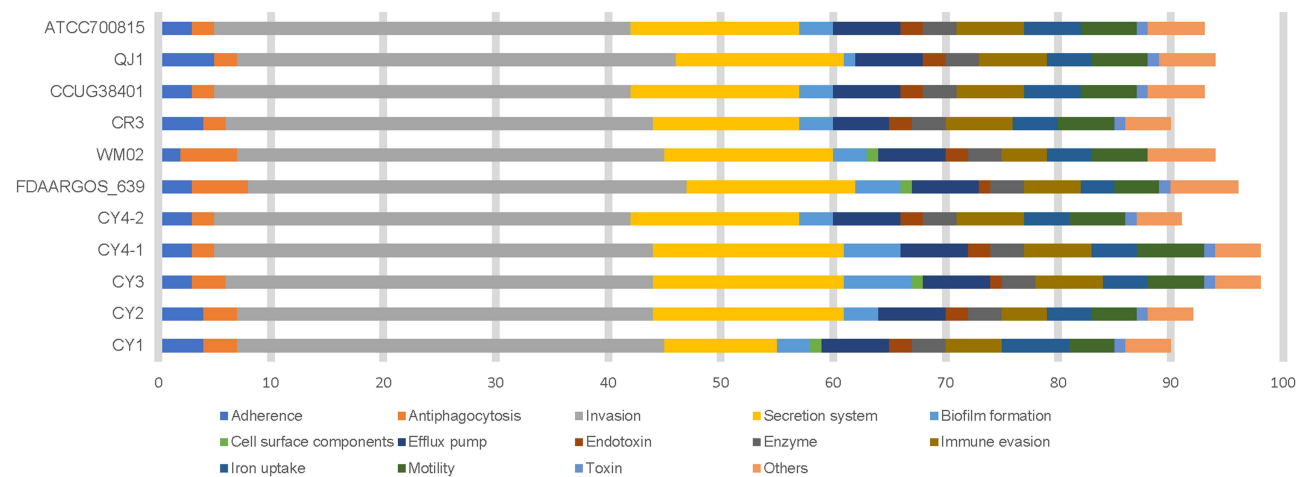
*C. gilardii* is an opportunistic pathogen that is widely present in the environment and has potential use in the detoxification of heavy metals and other biotechnological applications.<sup>24–27</sup> There are increasing case reports of infections caused by *C. gilardii*, even fatal cases.<sup>10</sup> However, the clinical analysis of *C. gilardii* infection is still relatively lacking. There are two clinical genomes available to date. One is *C. gilardii* strain FDAARGOS\_639 reported by the US Food and Drug Administration in 2020 (currently a representative genome), but with no clinical information. The other one is *C. gilardii* strain WM02 reported recently from an acute non-lymphocytic leukemia patient from China, also with no clinical information. To our knowledge, the four cases included in this study are the largest sample size so far for this rare infectious disease. We isolated and sequenced five strains from different body liquids, enriching the genetic data of *C. gilardii* and providing insight into the genetic differences between clinical and environmental strains. The clinical characteristics, treatment and outcome were explored to gain a deeper understanding of this disease.

Interestingly, three of the four patients got abscess (empyema and mediastinal abscess for the esophageal cancer patients and lung abscess for an immunocompetent patient) during the treatment, indicating there is a risk of purulent infection after *C. gilardii* infection. Abscess caused by *C. gilardii* infection has also been previously reported.<sup>12,13</sup> Tena et al reported a renal transplant recipient developed a muscular abscess caused by *C. gilardii*. Similarly, *C. gilardii* were





**B**



**Figure 3** The resistant and virulent gene comparison of multiple *C. gilardii* strains. **(A)** Gene mutations in resistant gene *gyrA*. The amino acids in mutated regions are shown, while non-mutated ones are hidden. Different amino acids are distinguished by different colors. **(B)** the virulence factor counts of *C. gilardii* strains. The VF categories were marked with different colors. The x-axis represents the number of genes, while the y-axis represents the strains.

isolated from the wound and blood culture in a case of pacemaker-associated infection. Thus, our study showed a representative clinical characteristic similar to that in other studies.

The mortality rate of our *C. gilardii* infection was also high. Thus, we compared the different characteristics between survivors and non-survivors. The results demonstrated that the values of IgG/IgM, CD4<sup>+</sup>/8<sup>+</sup> T cells, and CD19<sup>+</sup> B cells were lower in the fatal patients, suggesting the possibility of potential immunosuppression in deceased patients, which was first described by our group. Given the relatively small sample size, further studies are needed to determine the role of immunity in the *C. gilardii* infection.

Since several studies have reported unsatisfied clinical effect of multiple antibiotics,<sup>10–14</sup> we discussed the potential drug resistance genes based on the sequencing data. *C. gilardii* strains CY4-1/2 were genetically highly homologous to environmental isolates CR3 (tar pit, China), CCUG38401 (whirlpool, Sweden), and ATCC700815 (whirlpool, USA). Both carried the colistin resistance gene MCR-5.1, indicating that the source of infection in this patient may be a *C. gilardii* strain that is present in the environment. Besides, we found that almost half of the *C. gilardii* strains carried the MCR-5.1 gene, indicating that a high probability of acquiring the resistance gene through gene transfer.

*C. gilardii* is naturally resistant to a variety of antimicrobials,<sup>28</sup> and these intrinsic genes were also found in the present study, such as OXA-837, AAC (3)-IVb, and ANT (3)-Ib. In addition, we further confirmed that during the treatment process, the bacteria also acquired resistance to certain antimicrobials through gene mutation. In conclusion, *C. gilardii* has diverse antimicrobial resistance mechanisms.

We investigate the virulence of *C. gilardii*. Investigations on *C. gilardii* virulence factors are limited to the VFs counts and TTSS gene on individual trains. We conducted a screening of known and potential VFs in 10 strains, aiming to establish a correlation between the count of VFs and the clinically observable toxicity, particularly in the case of CY4-1 and CY4-2. However, despite our efforts, we have not yet identified a specific factor that plays a significant role in the virulence of *C. gilardii* among the numerous VFs assessed. Further assessments are required to unveil the underlying mechanism of virulence in *C. gilardii*.

At present, there are still difficulties in clinical identification and treatment of *C. gilardii*. The high-throughput sequencing method used here to study the clinically isolated *C. gilardii* strains can accurately identify its drug resistance genes and elucidate the mechanisms underlying acquired drug resistance, guiding antimicrobial treatment. Though the sample size in the present study was small and the genome assemblies are drafts, we successfully investigated the pathogenicity and drug resistance mechanisms. For the clinical treatment of *C. gilardii* infection, tetracyclines, ceftazidime, and cefepime are recommended first. Advanced antimicrobials such as meropenem and imipenem are not recommended.<sup>13,14,29</sup> Sequencing methods to identify resistance genes and predict drug resistance can guide antimicrobial treatment, reducing the abuse of antimicrobials and improving therapy.

## Conclusion

Collectively, our study represented four clinical cases of *C. gilardii* infection and conducted a high-throughput sequencing of the isolated *C. gilardii* strains. By identifying the clinical and genetic characteristics of this rare infectious disease, as well as the possible genes mediated the drug resistance, our work enhances the understanding of the disease and provides some directions for rational antimicrobial treatment. Further researches need to be performed to elucidate the pathogenic mechanism and the significant virulence genes of *C. gilardii* infection and to explore new therapeutic targets.

## Abbreviations

RICU, Respiratory Intensive Care Unit; SOFA, Sequential Organ Failure Assessment; APACHE II Acute Physiology And Chronic Health evaluation II.

## Data Sharing Statement

The draft assembled genomes of *C. gilardii* strains CY1, CY2, CY3, CY4-1, and CY4-2 have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database (GenBank accession numbers JAODVC000000000, JAODVD000000000, JAODVE000000000, JAODVF000000000, and JAODVG000000000). The raw sequencing reads were shared through NCBI-SRA with the bioproject IDs: PRJNA881018.

## Ethical Approval

The ethics committee of the affiliated Beijing Chao-Yang Hospital, Capital Medical University approved this study (2021-ke-501). This study was conducted in accordance with the Declaration of Helsinki. Written informed consent was waived due to the retrospective noninterventional design.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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