Letter to the Editor

Two Cases of Multi-antibiotic Resistant *Cronobacter* spp. Infections of Infants in China*

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Infections by *Cronobacter* spp. are hazardous to infants since they can lead to neonatal meningitis, bacteremia, and necrotizing enterocolitis. *Cronobacter* spp. are frequently resistant to β-lactam derivatives, macrolides, and aminoglycosides. In addition, multi-resistant strains have also been detected. In China, the isolation rate of *Cronobacter* spp. from commercial powdered infant formula (PIF) or follow-up formula (FUF) is relatively high. Nevertheless, clinical cases of *Cronobacter* infection have been ignored to date. Here we describe two cases of *Cronobacter* infection detected at the Wuhan Women and Children Medical Care Center Hospital (Wuhan City, China). We provide the genomic analysis of the isolates and the antibiotic-resistance profiles of the two strains. The *Cronobacter* strains identified in this study were not susceptible to third-generation cephalosporins, aminoglycoside, and/or trimethoprim-sulfamethoxazole. Whole genome sequencing revealed various genes known to encode antibiotic resistance. Future studies are needed to determine whether the genes predicted in this study are functional. As with *Enterobacter* spp., the antibiotic resistance of *Cronobacter* is a serious issue that requires more attention.

Key words: Multi-antibiotic resistant; *Cronobacter* spp.; Infant; Case report

Infections by *Cronobacter* spp. can result in a range of symptoms among different age groups. Compared with the adult population, a higher percentage of invasive infections occur among children less than five years of age[9]. These infections are hazardous to infants since they can lead to neonatal meningitis, bacteremia, and necrotizing enterocolitis (NEC). Based on multi-locus sequence typing (MLST), the genus *Cronobacter* consists of seven species, replacing the former single species *Enterobacter sakazakii* classification. There are many sequence types (STs) of *Cronobacter*. Many cases of neonatal meningitis are caused by *Cronobacter* spp. ST 4 of *C. sakazakii*[3]. The source of *Cronobacter* infections remains unclear since strains have been isolated from throat and sputum samples as well as from rectal and fecal swabs[1]. Although *Cronobacter* spp. exist widely in nature, including food, plants, water, and soil, as well as in housekeeping environments, they are recognized as a public health threat because they cause disease in cases of powdered infant formula (PIF) contamination.

Bacterial antibiotic resistance is a serious problem in treating hospital infections, thus surveillance of antibiotic susceptibility profiles is necessary. At the end of the 20th century, *Cronobacter* spp. were reported to be more sensitive to some antibiotics than other *Enterobacter* species[2]. Unfortunately, it was soon determined that *Cronobacter* spp. were resistant to broad-spectrum penicillins and to cephalosporins through the production of β-lactamases[5]. Recently, like other *Enterobacteriaceae*, *Cronobacter* spp. were frequently found to be resistant to β-lactam derivatives, macrolides, and aminoglycosides such as rifampicin, amoxicillin-clavulanic acid, streptomycin, tetracycline, or ampicillin. In addition, multi-resistant strains have also been detected[10]. The mechanism and source of their resistance to antibiotics are

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currently not well understood and are the focus of future studies.

In China, the isolation rate of *Cronobacter* spp. from commercial PIF or follow-up formula (FUF) samples is relatively high[8]. Nevertheless, clinical cases of *Cronobacter* infection have been ignored to date. This is the first report of two cases of *Cronobacter* infection of infants, identified at the Wuhan Women and Children Medical Care Center Hospital (Wuhan City, China). Here, we describe the cases, the genomic analysis of the isolates, and the antibiotic-resistance profiles of the two strains.

Case 1 Case 1 was a female infant born after 36 weeks of gestation (birth weight: 2900 g). Initially, the child appeared to be healthy and fed well on breast milk and supplementary PIF. 11 days after birth, she developed fever, muscle spasms in her extremities, and fed poorly. When admitted to hospital, her intracranial pressure was high and her spinal fluid contained numerous leukocytes. Treatment with intravenous cefoperazone, sulbactam sodium, meropenem, mannitol, and phenobarbital was started immediately. After a week, the infant was discharged from hospital and its mental and physical development was considered to be markedly impaired. *Cronobacter* was isolated from the cerebral spinal fluid (CSF) using selective medium (DFI agar). An API 20E kit was used for the presumptive identification of *Cronobacter*. Real-time PCR with primers for the macromolecular synthesis operon and MLST were performed for confirmation. By querying the MLST sequence type database for *Cronobacter* spp. (http://www.pubmlst.org/cronobacter/), the strain was identified as *C. malonaticus* ST 60. The strain was named Chcon_9 and was deposited in the *Cronobacter* MLST database. The pulse field gel electrophoresis (PFGE) pattern was compared with other patterns of the PulseNet China database, which contains strains (n = 252) mainly isolated from food, drinking water, and anal swabs. No matching pattern was found (Figure 1). This strain was tested for antibiotic susceptibility to 16 antibiotics using the broth dilution method and was found to be resistant to nine antibiotics including: ampicillin, azithromycin, ceftriaxone, chloramphenicol, doxycycline, gentamycin, tetracycline, trimethoprim-sulfamethoxazole, and sulfonamides. The minimal inhibitory concentration (MIC) values are shown in Table 1 and the results were interpreted using the *Enterobacteriaceae* data from the Clinical and Laboratory Standards Institute (CLSI). Whole genome sequencing was performed using an Illumina HiSeq 2500 sequencer and the assembled genome was compared with the Antibiotic Resistance Genes Database (ARDB; http://ardb.cbcb.umd.edu/index.html). Various genes known to encode antimicrobial resistance were identified within the genome sequence (Table 2). These whole genome results were deposited in DDBJ/EMBL/GenBank under the accession number LGRM00000000.

Table 1. Minimum Inhibitory Concentrations of 16 Antibiotics for *Cronobacter* in this Study

Antibiotics	MIC (μg/mL)	
	C. malonaticus HB03	*C. sakazakii* HB04
Ampicillin (AMP)	≥ 32	≥ 3
Azithromycin (AZI)	≥ 64	16
Amoxicillin/clavulanic acid 2:1 ration (AUG2)	4/2	4/2
Cefoxitin (FOX)	2	≥ 32
Cefepime (FEP)	4	≥ 32
Ceftriaxone (AXO)	≥ 8	≥ 8
Chloramphenicol (CHL)	≥ 64	≥ 32
Ciprofloxacin (CIP)	-	0.06
Doxycycline (DOX)	≥ 32	4
Gentamycin (GEN)	≥ 32	-
Imipenem (IMI)	-	-
Nalidixic acid (NAL)	2	4
Streptomycin (STR)	32	-
Sulfonamides (SMX)	≥ 512	≥ 512
Tetracycline (TET)	≥ 32	4
Trimethoprim-sulfamethoxazde (SXT)	≥ 4/75	0.25/4.75

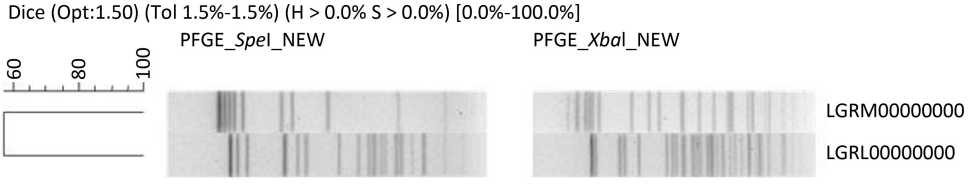

Figure 1. Dendrogram combining PFGE patterns of *Xba* I and *Spe* I digested DNA from *Cronobacter* spp. in this study.

Case 2 Case 2 was a female infant with no birth record details, as she was an abandoned baby living in a welfare house. She was fed with powdered-milk formula. When the baby was 10 days old, she had anoplasty because of an imperforate anus. After the operation, she started feeding poorly. Two months after birth, she developed anemia, diarrhea, moderate dehydration, and severe malnutrition. Although she was treated with anti-inflammatory drugs (intravenous meropenem), fluid replacement, and symptomatic treatments, her condition deteriorated. Finally, after 4 days in hospital, she died of circulatory and respiratory failure. *Cronobacter* was isolated from blood using DFI agar. The strain was identified using the same methods as in Case 1. The PFGE pattern did not match the strain isolated in Case 1, or any entries in the PulseNet China database (Figure 1). The MLST profile of the strain was identified as *C. sakazakii* ST 83. The strain was named Chcon_10 and was deposited in the *Cronobacter* MLST

Table 2. Putative Antibiotic Resistance Genes and Annotation of *Cronobacter* in this Study

Antibiotic Resistance	Putative Gene	Annotation	Species	
			C. malonaticus HB03	*C. sakazaakii* HB04
β-Lactam	*pbp1a*	The enzyme has a penicillin-insensitive	+	+
	pbp1b	tranlycosylase N-terminal domain and a	+	+
	pbp2	penicillin-insensitive transpeptidase C-teminal domain.	+	+
	ampC	Class A,B,C β-lactamase. This enzyme breaks	+	+
	ampA, ampR	theβ-lactam antibiotic ring open and	+	-
	bl3_l	deactivates the molecule's antibacterial	+	+
	*bl2be_*	properties.	-	+
	CTX-M-14		+	-
	bl2be_shv2			
Aminoglycosides	*aac3iia*	Aminoglycoside N-acetyltransferase, which modifies aminoglycosides by acetylation.	+	-
Macrolides	*aph3ia*	Aminoglycoside O-phosphotransferase, which	+	-
	aph33ib	modifies aminoglycosides by phosphorylation.	+	-
	aph6id		+	-
Tetracycline	*ant2ia*	Aminoglycoside O-nucleotidylyltransferase, which modifies aminoglycosides by adenylylation.	+	-
Phenicols	*erea*	Erythromycin esterase, which can inactivate erythromycin by lactone ring cleavage.	+	-
	tetd	Major facilitator superfamily transporter, tetracycline efflux pump.	+	-
	tet34	Xanthine-guanine phosphoribosytransferase.	+	+
	cata2	Group A chloramphenicol acetyltransferase, which can inactivate chloramphenicol.	+	-
Multidrug resistance efflux pump	*acra*		+	+
	acrb		+	+
	emrd		+	+
	emre		+	+
	macb	Multidrug resistance efflux pump	+	-
	mdtk		+	+
	mdth		+	+
	mdtg		+	+
	oprm		-	+
	tolc		+	+
	ykkc		+	+

database. The strain was resistant to the following six antibiotics: ampicillin, cefoxitin, ceftriaxone, cefepime, chloramphenicol, and sulfonamides (Table 1). Whole genome sequencing was performed as described in Case 1. The predicted antibiotic resistant genes are listed in Table 2. The whole genome results were deposited at DDBJ/EMBL/GenBank under the accession number LGRL00000000.

Seven species of *Cronobacter* have been identified, including *C. sakazakii*, *C. malonaticus*, *C. dublinensis*, *C. turicensis*, *C. muytjensii*, *C. universalis*, and *C. condiment*. However, *C. sakazakii* has mainly been reported to cause neonatal meningitis and NEC. In particular, *C. sakazakii* ST 4 has been more closely associated with neonatal meningitis[3]. In this study, the strain from Case 1 was *C. malonaticus* ST 60, which was firstly reported to cause neonatal meningitis. Although the other strain, identified as *C. sakazakii* ST 83, has been known to cause meningitis, it is uncommon in clinical practice. Contaminated PIF has been described as the primary cause of *Cronobacter* infection in feeding infants. However, we failed to trace the source of infection in these two cases, raising the possibility that there are other routes of transmission in *Cronobacter* infection.

Joshua B. Gurtler summarized the antibiotic resistance profile of *Cronobacter* infection and demonstrated that treatment should be guided by clinical judgment and *in vitro* antibiotic susceptibility testing[2]. Since then, although antibiotic resistance in clinical isolates has been reported less frequently, antibiotic resistance in isolates from food and the environment has been observed[7]. The most common antibiotics that *Cronobacter* spp. from food and the environment are resistant to were cephalothins and penicillins[10] , in agreement with other reports[5]. The reason for this is that β-lactamase production appears to be more widespread among isolates of *Cronobacter* spp.[5]. Of the 16 antibiotics tested in this study, the two isolates were susceptible to amoxicillin/clavulanic acid, ciprofloxacin, imipenem, nalidixic acid, and streptomycin. Because antibiotic use is restricted in infants, carbapenems, such as imipenem, were actually the only choice for treatment. Multi-antibiotic resistance is common in *Enterobacter* spp., but rare in *Cronobacter*. Here, two cases with multi-antibiotic resistance are presented, indicating that there is a similar problem of antibiotic resistance for *Cronobacter*.

Whole genome sequencing identified the presence of various genes known to encode antibiotic resistance proteins. The presence of some of these genes, such as *ampC*, *ampA*, *ampR*, *bl3_l*, *bl2be_CTX-M-14*, and *bl2be_shv2* is predicted to be related to β-lactamase-mediated antibiotic resistance. The unusual resistance phenotype in *C. sakazakii* and *C. malonaticus* has been attributed to AmpC β-lactamases[7]. Some *Enterobacteriaceae*, such as *Serratia marcescens*, *Citrobacter freundii*, *Providencia* spp. and *Morganella morganii*, that possess chromosomally determined AmpC β-lactamases, may express the enzymes at a high level following exposure to β-lactams. However, the risk of clinical failure using β-lactams that show susceptibility *in vitro* is less clear in these species than in *Enterobacter* spp.[4]. Although *bl2be_CTX-M-14* and *bl2be_shv2* have not been found in *Cronobacter* previously, the presence of *CTX-M-15* and *SHV-12* in *Cronobacter* has been reported[6]. In this study, many genes encoding multidrug resistance efflux pumps were screened. Bacterial multidrug resistance efflux pumps constitute an important mechanism of antibiotic resistance and are required by many pathogens for successful infection. For different antibiotics, different genes in the ARDB were found to be relevant (Table 2). Further studies are needed to determine whether the genes predicted in this study are functional.

This report highlights the importance of antibiotic resistance in two *Cronobacter* species that are known to cause life-threatening infections in infants. Treatment of *Cronobacter* infections with carbapenems or the newer third-generation cephalosporins in combination with an aminoglycoside or trimethoprim-sulfamethoxazole is recommended[6]. However, *Cronobacter* was not susceptible to third-generation cephalosporins, aminoglycoside, and trimethoprim-sulfamethoxazole in this study. As with *Enterobacter* spp., the antibiotic resistance of *Cronobacter* is a serious issue that needs more attention. Clinical studies that clearly define optimal treatment for *Cronobacter* spp. are required.

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