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Antibiotic Susceptibility of *Cronobacter* spp. Isolated from Clinical Samples

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Abstract

Cronobacter spp. have been recognized as causative agents of various severe infections in pre-term or full-term infants as well as elderly adults suffering from serious underlying disease or malignancy. A surveillance study was designed to identify antibiotic resistance among clinical *Cronobacter* spp. strains, which were isolated from patients of two hospitals between May 2007 and August 2013. Altogether, 52 *Cronobacter* spp. isolates were analyzed. Although MALDI-TOF mass spectrometry recognized all *Cronobacter sakazakii* and *Cronobacter malonaticus* strains, it could not identify *Cronobacter muytjensii* strain. Nevertheless, all strains were identified as *Cronobacter* spp. using multilocus sequence typing (MLST). Strains were tested against 17 types of antibiotics, using the standard microdilution method according to the 2018 European Committee on Antimicrobial Susceptibility Testing criteria. Three *Cronobacter* species were identified as *C. sakazakii* (n = 33), *C. malonaticus* (n = 18), and *C. muytjensii* (n = 1); all isolates were susceptible to all tested antibiotics. All strains were PCR-negative for bla_{TEM}, bla_{SHV}, and bla_{CTX-M} β-lactamase genes, as well. Even though the results of this study showed that *Cronobacter* spp. isolates were pan-susceptible, continued antibiotic resistance surveillance is warranted.

Key words: antibiotics, antimicrobial susceptibility, *Cronobacter* spp., MALDI-TOF mass spectrometry, multilocus sequence typing

Introduction

Cronobacter is a genus of Gram-negative, facultative-anaerobic, nonspore-forming, motile bacteria belonging to the *Enterobacteriaceae* family. At present, seven species are known: *Cronobacter sakazakii*, *C. malonaticus*, *C. dublinensis*, *C. muytjensii*, *C. turicensis*, *C. condimenti*, and *C. universalis* (Iversen et al. 2007; Iversen et al. 2008; Joseph et al. 2012; Stephan et al. 2014). Except for *C. condimenti*, all species of *Cronobacter* have been isolated from clinical specimens. The *Cronobacter* species of serious clinical significance are as follows: *C. sakazakii*, *C. malonaticus*, *C. turicencis*, and *C. universalis*. Other members of the genus (*C. dublinensis*, *C. muytjensii*, and *C. condimenti*) are primarily environmental commensals with low clinical significance (Iversen et al. 2007; Kucerova et al. 2010; Holy et al. 2011; Holy et al. 2014; Holy and Forsythe 2014; Forsythe 2018). *Cronobacter* spp. are opportunistic pathogens that cause rare but life-threatening diseases such as meningitis, necrotizing enterocolitis, and bloodstream infections in neonates and infants. The infections caused by these bacteria are often severe with fatal health consequences. The lethality rate of meningitis in infants was estimated to be 41.9%

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with death occurring within hours after the manifestation of symptoms (Willis et al. 1988; Friedemann 2009; Holy and Forsythe 2014). The surviving individuals usually develop irreversible sequelae including serious neurological complications such as quadriplegia and impaired mental development (Bowen and Braden 2006). Infants up to two months of age, premature with low birth weight or immunocompromised newborns are at the highest risk for infection. *Cronobacter* spp. have also been recognized as causative agents of various infections in elderly adults suffering from serious underlying disease or malignancy (Dennison and Morris 2002; See at al. 2007).

Cronobacter spp. are naturally resistant to all macrolides, lincomycin, clindamycin, streptogramins, rifampicin, fusidic acid, and fosfomycin. Infections caused by these bacteria are usually treated with various combinations of ampicillin, gentamicin, cefotaxime, and chloramphenicol (Muytjens et al. 1983; Biering et al. 1989; Bar-Oz et al. 2001; Block et al. 2002). *Cronobacter* spp. tend to be more sensitive to most antibiotics that are being used clinically to treat infections caused by *Enterobacteriaceae*, although resistance to ampicillin has developed (Muytjens and van der Ros-van de Repe 1986). In 1980, all tested strains were susceptible to ampicillin, whereas in 2001, five cases of *Cronobacter* infection in which one or more of the isolates were resistant to ampicillin and first- and second-generation cephalosporins were described (Farmer et al. 1980; Lai 2011). Similarly, Block et al. (2002) reported that all *Cronobacter* isolates tested were β-lactamase positive. Caubilla-Barron et al. (2007) previously reported two neonatal deaths from extended-spectrum β-lactamase (ESBL)-encoding *C. sakazakii* strains in a retrospective study of the *Cronobacter* necrotizing enterocolitis and meningitis outbreak in the neonatal intensive care unit. Then, Stock and Weidemann (2002) studied some *Cronobacter* strains and found that all strains were susceptible to the tested β-lactams.

The β-lactamase activity in *Cronobacter* has been frequently reported by others (Pitout et al. 1997; Caubilla-Barron et al. 2007; Baldwin et al. 2009). In 1997, a low-level β-lactamase production in *Cronobacter* was detected by Pitout et al. (1997). In addition, Lai (2001) also reported that *Cronobacter* strains were resistant to ampicillin, cefazolin, and extended-spectrum penicillin. Moreover, a recent study showed that about 10.2% of the *Cronobacter* strains were resistant to cefotaxime, which is one of the third-generation cephalosporins related to penicillin (Pan et al. 2014). Rising antimicrobial resistance is generally a public health concern, potentially leading to prolonged illness and a higher risk of mortality.

The aim of this study was to examine over time the changes in antibiotic resistance of different *Cronobacter* spp. strains and to determine the susceptibility of *Cronobacter* isolates from clinical samples to 17 types of antibiotics.

Experimental

Materials and Methods

Bacterial strains and cultivation. The 52 isolates were collected during a survey of *Cronobacter* spp. carriage in patients of two hospitals over the 6-year period from May 2007 to August 2013. All strains were isolated from clinical samples (Table I). Microorganisms were routinely cultivated on blood agar (Oxoid, UK) at temperature of 37°C overnight.

Identification of *Cronobacter* spp. by MALDI-TOF MS. *Sample preparation*. For the protein extraction three colonies of each microorganism grown overnight on blood agar plates at 37°C under aerobic conditions were picked and resuspended in 300 µl of water (Sigma-Aldrich, USA) in 1.5 ml tube (Eppendorf, Germany). Then, 900 µl of absolute ethanol were added and mixed. The tubes were centrifuged twice at 13 000 rpm for 2 min and the supernatant was removed. The pellet was kept for 15 min at room temperature. The pellet was then dissolved in 50 µl of 70% formic acid. Finally, an equal volume of acetonitrile was added to the mixture and the mixture was stirred and centrifuged at 13 000 rpm for 2 min. The mixture was then vortexed for 15 seconds. After this, 1 µl of the supernatant was spotted eight times onto a MALDI target, followed by the addition of 1 µl of HCCA (alpha-cyano-4-hydroxy-cinnamic acid made as saturated solution in 30% acetonitrile/0.1% trifluoroacetic acid) and allowed to dry at room temperature before MALDI-TOF MS analysis. Immediately after drying, the spot was overlaid with 1 µl of matrix (Schulthess et al. 2016). MALDI-TOF MS measurements were performed with a microflex LT (Bruker Daltonics, Bremen, Germany). Each spot was analysed in triplicate (24 spectra in total). Each generated spectrum resulted from 240 laser shots (40 laser shots at six multiple positions on the target spot). All investigations were performed in triplicate.

Data analysis. The resulting 72 spectra were carefully analysed using the Flex Analysis (version 3.0, Bruker Daltonics, Germany) and subjected to smoothing and baseline subtraction. Furthermore, at least 24 spectra of each strain were used to create a single standard mass spectrum (MSP) with the default setting of the BioTyper MSP creation method. To visualize the difference between the strains, MSP dendrogram clustering was constructed using the standard settings in MALDI BioTyper™ software.

Table I
Identification and genotyping of *Cronobacter* spp. isolated from clinical samples.

Strain	Strain No.	Log (score)*	Sequence type	Origin
C. sakazakii	1836	2.354	4	Wound swab
C. sakazakii	1837	2.432	4	Wound swab
C. sakazakii	1839	2.503	4	Smear from area of percutaneous endoscopic gastrostomy
C. sakazakii	1840	2.452	4	Sputum
C. sakazakii	1841	2.444	4	Sputum
C. sakazakii	1842	2.409	4	Sputum
C. sakazakii	1901	2.467	4	Sputum
C. sakazakii	1902	2.421	4	Sputum
C. sakazakii	1903	2.487	4	Sputum
C. sakazakii	1915	2.509	4	Sputum
C. sakazakii	1916	2.464	4	Sputum
C. sakazakii	1996	2.484	4	Sputum
C. sakazakii	1997	2.505	4	Sputum
C. sakazakii	1998	2.485	4	Sputum
C. sakazakii	2000	2.479	4	Rectal swab
C. sakazakii	2001	2.473	4	Swab of the oral cavity
C. sakazakii	2002	2.476	4	Sputum
C. sakazakii	2003	2.423	4	Sputum
C. sakazakii	2005	2.457	4	Sputum
C. sakazakii	2006	2.357	4	Sputum
C. sakazakii	2007	2.438	4	Sputum
C. sakazakii	2008	2.416	4	Sputum
C. sakazakii	2009	2.459	4	Tongue swab
C. sakazakii	2010	2.388	4	Throat swab
C. sakazakii	2011	2.417	4	Suction catheter
C. sakazakii	2012	2.494	4	Sputum
C. sakazakii	2013	2.451	4	Sputum
C. sakazakii	2021	2.446	4	Sputum
C. sakazakii	2016	2.448	4	Sputum
C. sakazakii	2017	2.510	4	Sputum
C. sakazakii	2019	2.367	4	Sputum
C. sakazakii	2022	2.426	4	Sputum
C. sakazakii	1995	2.508	64	Sputum
C. malonaticus	1826	2.313	7	Cannula
C. malonaticus	1827	2.293	7	Cannula
C. malonaticus	1828	2.419	7	Nose swab
C. malonaticus	1829	2.329	7	Throat swab
C. malonaticus	1830	2.429	7	Throat swab
C. malonaticus	1831	2.415	7	Throat swab
C. malonaticus	1832	2.373	7	Throat swab
C. malonaticus	1833	2.398	7	Stool - dissection
C. malonaticus	1834	2.345	7	Throat swab
C. malonaticus	1835	2.336	7	Throat swab
C. malonaticus	1914	2.426	7	Sputum
C. malonaticus	1917	2.387	7	Throat swab
C. malonaticus	1999	2.361	7	Throat swab
C. malonaticus	2004	2.343	7	Throat swab

Table I. Continued.

Strain	Strain No.	Log (score)*	Sequence type	Origin
C. malonaticus	2014	2.388	7	Throat swab
C. malonaticus	2015	2.353	7	Throat swab
C. malonaticus	2018	2.339	7	Sputum
C. malonaticus	2020	2.173	7	Stool
P. agglomerans/ *C. muytjensii*	1838	2.358	28	Sputum

* Log (score) according to MALDI-TOF identification. According to the MALDI-TOF MS strain No. 1838 was identified as *P. agglomerans*.
This strain was subsequently reclassified as *C. muytjensii* according to results of the MLST

The linkage function was normalised according to the distance between 0 (perfect match) and 1 000 (no match). Species with distance levels under 500 have been described as reliably classified species (Sauer et al. 2008).

Following the creation of MSPs of strains, each MSP was identified. All spectra were imported into the Biotyper software and analyzed against the Bruker library. Identification scores of ≥ 2.0 have been declared by manufacturer as a reliable identification to the species level and scores of ≥ 1.7 but < 2.0 to the genus level if the conditions of species and genus consistency are fulfilled, respectively. Isolates with log-score above 2.3 were used for dendrogram generation if the best three matches indicated the same species.

Multilocus sequence typing (MLST). The MLST scheme for the strains investigated was performed previously by Alsonosi et al. (2015) and published in the curated *Cronobacter* PubMLST open access database (http://www.pubmlst.org/cronobacter). The seven housekeeping genes *atpD* (ATP synthase beta chain), *fusA* (elongation factor G), *glnS* (glutaminyl-tRNA synthetase), *gltB* (glutamate synthase large subunit), *gyrB* (gyrase subunit B), *infB* (translation initiation factor IF-2), and *ppsA* (phosphoenolpyruvate synthase) were sequenced. The results obtained were compared with data at the *Cronobacter* PubMLST database.

Antimicrobial susceptibility testing. All *Cronobacter* strains were tested for susceptibility against 17 types of antibiotics, that were: ampicillin, aztreonam, ampicillin/sulbactam, gentamicin, piperacillin, tobramycin, piperacillin/tazobactam, amikacin, cefuroxime, ciprofloxacin, cefotaxime, tigecycline, ceftazidime, colistin, cefepime, trimethoprim/sulfamethoxazole, and meropenem. The susceptibility to antibiotics was assessed by a standard microdilution method according to the European Committee on Antimicrobial Susceptibility Testing criteria 2018 (EUCAST 2018).

Detection of *blaTEM*, *blaSHV*, and *blaCTX-M* genes. The detection of *blaTEM*, *blaSHV*, and *blaCTX-M* genes (encoding TEM, SHV, and CTX-M types of β-lactamases) was performed by PCR in all *Cronobacter* spp. isolates examined. As positive controls, the following strains were used: *Escherichia coli* NCTC 13400 ($blaCTX\text{-}M_{-15}$), *E. coli* NCTC 13351($blaTEM_{-3}$) and *Klebsiella pneumoniae* NCTC 13368 ($blaSHV_{-18}$). Total bacterial DNA was isolated from an overnight culture of the isolates (16 h, 37°C) grown on meat-peptone agar. Two colonies were suspended in 100 µl of water and heated at 95°C for 10 min. After removing of cellular debris by centrifugation at 13 000 × *g* for 2 min, the supernatant was used as a template DNA for amplification. Polymerase chain reaction with a specific set of primers was performed for the detection of genes that encode relevant beta-lactamases (Arlet et al. 1995; Chanawong et al. 2000; Pagani et al. 2003).

Results

Identification and typing of *Cronobacter* isolates. Bacterial strains were identified by the MALDI-TOF mass spectrometry and genotyped by MLST. The protein spectra of 52 isolates were compared with those included in MALDI BioTyper™ reference database. The matching of an unknown spectrum with that in reference database was quantified by the log-score. The protein spectra of 52 isolates were obtained from three independent assays. The logarithms of score values ≥ 2 were required for reliable identification of the unknown strains at the species level. The average values of the log-score fall within limits of 2.173 to 2.510 (Table I). The most prevalent species recovered from clinical samples were *C. sakazakii* (n = 33; 63.5%) and *C. malonaticus* (n = 18; 34.6%). Only one strain was identified as *Pantoea agglomerans* (1.9%) – the strain No. 1838. However, this strain was subsequently reclassified as *C. muytjensii* according to results of the MLST.

As we previously published, based on the MLST results for clinical strains, the intra-species diversity was poor. A majority of *C. sakazakii* strains (n = 32) belonged to the dominant sequence type 4, whereas only one *C. sakazakii* strain isolated from sputum was

determined as ST64. All *C. malonaticus* isolates were classified as ST7. Only *C. muytjensii* was assigned to ST28 (Alsonosi et al. 2015).

Antibiotic susceptibility. Antibiotic resistance profiling revealed that all 52 isolates were sensitive to ampicillin, aztreonam, ampicillin/sulbactam, gentamicin, piperacillin, tobramycin, piperacillin/tazobactam, amikacin, cefuroxime, ciprofloxacin, cefotaxime, tigecycline, ceftazidime, colistin, cefepime, trimethoprim/sulfamethoxazole, and meropenem. No resistant strains were found. The minimal inhibitory concentrations of the antimicrobial mentioned above were given in Table II. The bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$, β-lactamase genes in the genome of our isolates were not found in any *Cronobacter* species, as well.

Discussion

The *Enterobacteriaceae* family are often associated with hospital-acquired infections. The most vulnerable population consists of immunocompromised patients (Holy et al. 2012; Matouskova et al. 2012; Matouskova and Holy 2013; Matouskova and Holy 2014). Antimicrobial resistance is a public health concern because it may cause failure of conventional treatment, resulting in prolonged illness and a higher risk of mortality. Resistance among human pathogens of *Enterobacteriaceae* has direct clinical impact on patients due to longer treatment and hospitalization, need for more expensive drugs, and often fatal consequences. Thus, infections due to resistant *Klebsiella* spp., *E. coli*, and *Enterobacter* spp. are now known as a major problem associated with health-care associated infections. The *Cronobacter* genus has undergone an extensive diversification during the course of its evolution, with some species clearly pathogenic for humans and other species still with unknown or uncertain impact on human health. Unfortunately, information on the diversity, pathogenicity, and virulence of *Cronobacter* species obtained from various sources is still relatively scarce and fragmentary. Applying the MLST scheme on clinical *C. sakazakii* strains revealed that the strains associated with meningitis, bacteremia, undefined infection, or necrotizing enterocolitis in neonates, infants, and children belong to the predominant sequence type profile 4 (Joseph and Forsythe 2011; Hariri et al. 2013). Virulence-related traits have also been found through draft and complete genome sequencing of *Cronobacter* spp. (Bar-Oz et al. 2001; Stephan et al. 2014). In some studies, up to 50% of *Cronobacter* spp. infection in adults had underlying malignancy (Lai 2001).

Two distinct identification systems, MALDI-TOF mass spectrometry and MLST, were compared in this study. MALDI-TOF MS protein profiling, based on the protein spectra obtained from intact whole cells, cell lysates, or bacterial extracts, is now widely used as an instrumental technique for bacterial identification. The main advantage of this analytical method, compared with other typing procedures, is its rapidity, low-cost requirements, and ease of use. Although the method usually provides the reliable discrimination of the common human pathogens (Barbuddhe et al. 2008; Mellmann et al. 2008; Davies et al. 2012; Kuhns et al. 2012), the correct identification of unknown protein profiles depends on the size and precision of the default database. In our case, the majority of isolates was reliably discriminated at the species level in congruence with results obtained via MLST. Only isolate 1838 was misidentified as *Pantoea agglomerans* by MALDI-TOF MS. Further analysis by MLST resulted in a re-classification of this microorganism as *C. muytjensii*, sequencing type 28 (Table I). An application of at least two independent identification methods is therefore recommended to minimize the number of misidentified strains.

Multilocus sequence typing divided 52 clinical strains into three species and five sequencing types (Alsonosi et al. 2015, Table I). Nearly all of the 33 *C. sakazakii* were typed as neonatal ST4, and only one was classified as a neonatal ST64. The 18 *C. malonaticus* strains belonged to ST7, which is usually isolated and causes serious infections in neonates or adults. The spectrum of *Cronobacter* spp. and types is quite similar to those reported in previous studies (Joseph and Forsythe 2011; Kadlicekova et al. 2018). Poor species and intraspecies diversity of the strains obtained from the clinical samples indicates that mucosa of human beings may be colonized by only limited *Cronobacter* sequence types.

Antimicrobial susceptibility was performed against 17 antibacterial agents. It is known that the extensive use of antimicrobials in agriculture and health-care facilities has led to the emergence of resistant bacterial strains. Nonetheless, the present study has shown that all used clinical strains of *Cronobacter* were susceptible to 17 antimicrobial agents, which represent a broad spectrum of typical antibiotics for enterobacteria. Similarly to these study findings, other studies have reported the susceptibility of *Cronobacter* strains to all antibiotics that were used in these studies (Terragno et al. 2009; Hochel et al. 2012; Vojkovska et al. 2016; Brandão et al. 2017). In these studies, all *Cronobacter* strains were isolated from food sources or powered infant formula and mainly *C. sakazakii* was investigated. Therefore, our study is a unique one since it shows a susceptibility of clinical isolates of both species *C. sakazakii* and *C. malonaticus*. These two species have been found to be associated with several serious neonatal and adult infections (See et al. 2007; Healy et al. 2010; Asato et al. 2013; Hariri et al. 2013; Brandão et al. 2017; Alsonosi

Table II
Minimum inhibitory concentration (MIC) of *Cronobacter* spp. isolated from clinical samples.

Strain	Strain No.	Minimum inhibitory concentration – MIC (mg · l^{-1})																
		AMP	AMS	PIP	PPT	CRX	CTX	CTZ	CPM	MER	AZT	GEN	TOB	AMI	CIP	TIG	COL	COT
C. sakazakii	1836	1	2	1	1	4	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	0.5	1
C. sakazakii	1837	0.5	0.5	1	1	1	0.1	0.1	0.1	0.1	0.2	0.2	0.5	2	0.1	0.06	0.5	1
C. sakazakii	1839	1	1	1	1	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	0.5	1
C. sakazakii	1840	1	1	1	1	4	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	1	1
C. sakazakii	1841	1	2	1	1	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	0.5	1
C. sakazakii	1842	1	2	1	1	4	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	0.5	1
C. sakazakii	1901	0.5	0.5	2	1	1	0.1	0.1	0.2	0.1	0.2	0.2	0.5	0.5	0.1	0.2	0.5	1
C. sakazakii	1902	0.5	0.5	2	1	1	0.1	0.1	0.2	0.1	0.2	0.2	0.5	0.5	0.1	0.1	0.5	1
C. sakazakii	1903	1	0.5	2	1	2	0.1	0.1	0.2	0.1	0.2	0.2	0.5	0.5	0.1	0.1	0.5	2
C. sakazakii	1915	0.5	0.5	2	1	0.5	0.1	0.1	0.2	0.1	0.2	0.2	0.5	0.5	0.1	0.1	0.5	1
C. sakazakii	1916	8	2	8	4	4	0.1	0.5	0.1	0.1	0.2	0.2	0.5	1	0.1	0.1	0.5	1
C. sakazakii	1996	4	1	4	1	1	0.1	0.1	0.1	0.5	0.2	0.2	0.5	2	0.06	0.2	0.5	1
C. sakazakii	1997	1	0.5	2	1	1	0.1	0.1	0.2	0.1	0.2	0.2	0.5	0.5	0.1	0.06	0.5	1
C. sakazakii	1998	0.5	0.5	2	1	1	0.1	0.1	0.2	0.1	0.2	0.2	0.5	0.5	0.1	0.1	0.5	1
C. sakazakii	2000	1	1	1	1	1	0.1	0.1	0.1	0.1	0.2	0.2	0.5	2	0.2	0.06	0.5	0.2
C. sakazakii	2001	2	1	2	1	4	0.1	0.1	0.1	0.5	0.1	1	0.2	1	0.06	0.03	0.1	0.2
C. sakazakii	2002	1	1	1	1	0.5	0.1	0.1	0.1	0.1	0.2	0.2	0.5	2	0.2	0.06	0.5	0.2
C. sakazakii	2003	2	1	2	1	4	0.1	0.1	0.1	0.5	0.1	1	0.2	2	0.06	0.06	0.1	0.2
C. sakazakii	2005	2	1	2	1	4	0.1	0.1	0.1	0.5	0.1	1	0.2	1	0.06	0.03	0.1	0.2
C. sakazakii	2006	2	0.5	2	1	4	0.1	0.1	0.1	0.5	0.1	1	0.5	1	0.06	0.03	0.1	0.2
C. sakazakii	2007	2	1	2	1	4	0.1	0.1	0.1	0.5	0.1	1	0.2	1	0.06	0.03	0.1	0.5
C. sakazakii	2008	2	1	2	1	4	0.1	0.1	0.1	0.5	0.1	1	0.5	1	0.06	0.03	0.1	0.2
C. sakazakii	2009	0.5	0.5	2	1	1	0.1	0.1	0.2	0.1	0.2	0.2	0.5	0.5	0.1	0.1	0.5	0.2
C. sakazakii	2010	2	1	2	1	2	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	1	2
C. sakazakii	2011	2	1	2	1	2	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	0.2	2
C. sakazakii	2012	1	0.5	2	1	1	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	0.2	2
C. sakazakii	2013	1	1	2	1	2	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	0.5	2
C. sakazakii	2021	2	1	2	1	2	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	1	2
C. sakazakii	2016	2	1	2	1	1	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	0.5	2
C. sakazakii	2017	2	1	2	1	2	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	1	2

Table II. Continued.

Strain	Strain No.	Minimum inhibitory concentration – MIC (mg · l^{-1})																
		AMP	AMS	PIP	PPT	CRX	CTX	CTZ	CPM	MER	AZT	GEN	TOB	AMI	CIP	TIG	COL	COT
C. sakazakii	2019	2	1	2	1	2	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	1	2
C. sakazakii	2022	2	1	2	1	4	0.1	0.1	0.1	0.5	0.1	1	0.2	1	0.06	0.03	0.1	0.5
C. sakazakii	1995	4	2	2	1	4	0.1	0.1	0.1	0.5	0.2	0.2	0.5	1	0.06	0.2	1	1
C. malonaticus	1826	0.5	1	1	1	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	1	1
C. malonaticus	1827	0.5	1	1	1	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	1	1
C. malonaticus	1828	0.5	1	1	1	1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.5	0.03	0.06	1	1
C. malonaticus	1829	0.5	1	1	1	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	1	1
C. malonaticus	1830	1	2	1	1	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	1	1
C. malonaticus	1831	1	1	1	1	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	0.1	1
C. malonaticus	1832	0.5	1	1	1	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	1	1
C. malonaticus	1833	0.5	1	1	1	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	1	1
C. malonaticus	1834	0.5	1	1	1	1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	1	1
C. malonaticus	1835	0.2	1	1	1	1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	1	0.03	0.06	1	1
C. malonaticus	1914	2	2	1	1	4	0.1	0.1	0.1	0.5	0.1	0.2	0.5	1	0.06	0.5	0.2	1
C. malonaticus	1917	1	1	1	1	1	0.1	0.1	0.1	0.5	0.1	0.2	0.2	0.5	0.06	0.2	0.2	1
C. malonaticus	1999	1	0.5	2	1	1	0.1	0.1	0.1	0.5	0.2	0.2	0.2	1	0.06	0.2	1	1
C. malonaticus	2004	0.5	0.5	2	1	1	0.1	0.1	0.1	0.1	0.2	0.2	0.5	0.5	0.1	0.1	0.5	0.2
C. malonaticus	2014	1	0.5	2	0.5	2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.06	0.2	2	2
C. malonaticus	2015	1	0.5	2	1	1	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	1	2
C. malonaticus	2018	1	1	2	1	2	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	1	2
C. malonaticus	2020	1	0.5	2	1	0.5	0.1	0.2	0.1	0.5	0.2	0.5	0.5	1	0.1	0.06	1	2
P. agglomerans/ *C. muytjensii*	1838	1	2	1	1	1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	1	0.03	0.06	0.2	1

AMP – ampicillin; AZT – aztreonam; AMS – ampicillin/sulbactam; GEN – gentamicin; PIP – piperacillin; TOB – tobramycin; PPT – piperacillin/tazobactam; AMI – amikacin; CRX – cefuroxime; CIP – ciprofloxacin; CTX cefotaxime; TIG – tigecycline; CTZ – ceftazidime; COL – colistin; CPM – cefepime; COT – trimethoprim/sulfamethoxazole; MER – meropenem
MIC (Minimum inhibitory concentration) – the lowest concentration of the antibiotics that can inhibit effectively growth of the tested microorganism

et al. 2018). As a part of the present study, β-lactamase genes, bla_{TEM}, bla_{SHV}, and bla_{CTX-M}, were screened among *Cronobacter* isolates. The results were consistent with the results of susceptibility assays, as all strains were negative for β-lactamase genes. However, other studies have shown emergence of resistance to penicillin and extended-spectrum β-lactamases (Block et al. 2002; Caubilla-Barron et al. 2007). Recently, two studies have shown that multi-drug resistance in *Cronobacter* is emerging through horizontal gene transfer mechanisms (Cui et al. 2015; Shi et al. 2016). In addition, the excessive use of antibiotics in the hospital environment facilitates the possibility of emerging of a high level of antimicrobial resistance among clinical strains. Therefore, the surveillance studies would assist in prevention of the developing this phenomenon and in keeping *Cronobacter* susceptible to the majority of the available clinical antimicrobial agents.

Conclusions

A low level of antibiotic resistance among *Cronobacter* spp. in our region is due to a good antibiotic usage policy. The minimizing of the occurrence of encephalitis, necrotizing enterocolitis, and septicemia among infants in the Czech Republic caused by *Cronobacter* spp. is also due to the excellent hospital care. We were able to isolate only 52 strains during the 6-year period of our study, and the majority of them were isolated from adults.

This study shows that although antimicrobial resistance among *Cronobacter* spp. is not a big issue nowadays, many resistant cases among *Cronobacter* spp. have been reported worldwide. Increasing antimicrobial resistance is not only a clinical problem associated with health-care facilities, but it is also a big public health issue. Selection pressure plays a crucial role in their spreading not only in health care facilities but also in the environment (e.g., food chains and waste waters).

The authors are aware of the limitations associated with this study, especially of the low number of samples. On the other hand, the advantage is that these samples were collected over quite a long period of time.

No official breakpoints by EUCAST were established for *Cronobacter* spp., only for *Enterobacteriaceae* in general, which is not sufficient. The epidemiological cut-off value (ECOFF) for *Cronobacter* spp. should be established in the near future.

Author's contributions

OH, AA, IH, MK, AA, SF participated in the study design, coordination and carried out data analyses. MR, SZ, PM, JP, DC participated and performed measurements, laboratory testing's and data collection. All authors read and approved the final manuscript. All authors contributed to the draft of the manuscript and discussed results. All authors gave final approval for publication.

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Conflict of interest

Author does not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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