

Study of Antimicrobial Susceptibility And Biofilm Formation of Cronobacter Sakazakii Isolates From Neonatal Sepsis In Southwest Iran

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Research

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Abstract

Background: The Cronobacter genus is a member family of the Enterobacteriaceae. The isolates of *C. sakazakii* have been suggested to be responsible for fatal neonatal infections, which gives rise to sepsis, necrotizing enterocolitis, and meningitis, with a high mortality rates. The aim of the present study was to investigate the antimicrobial susceptibility and biofilm formation of *C. sakazakii* isolates from neonatal sepsis in Southwestern Iran.

Results: During the period of study, 734/1045 bacterial positive growth samples were collected from patients. Overall, from 734 bacterial positive growth samples 120 isolates were *C. sakazakii* based on culture, biochemical tests and PCR amplification. seventy-four (61%) neonates had primary sepsis and (33%) had late sepsis. Regarding birth bodyweight, (22%) neonate weighted below 1000 gr, 61 (50%) between 1500 and 2500, and (26%) more than 2500 gr. In case of *C. sakazakii* isolates, the highest resistance rates belonged to Ampicillin (70%), followed Amoxicillin (%59) and Ampicillin/sulbactam (83%). However, *C. sakazakii* had low levels resistance to cefepime and tetracycline. In total, of the 120 isolated bacteria, (70%) were biofilm producers, of which, (37%) produced strong biofilms, (15%) produced moderate biofilms, (17%) were weak biofilm producers and (28%) were not biofilm producers.

Conclusion: Taken together, the high rate of *C. sakazakii* in neonates was high in the NICU. Age, higher birthweight, and caesarian delivery were the most remarkable risk factors for *C. sakazakii*. The majority of *C. sakazakii* strains were hospital-associated, which is the indication of NICU admission patterns. Our findings suggest that the active surveillance of neonates for *C. sakazakii* is required to be considered as a part of strategies to detect importation and prevent transmission of *C. sakazakii* within the NICU.

Background

The Cronobacter genus is a member family of the Enterobacteriaceae and includes seven species, namely *C. sakazakii*, *C. malonaticus*, *C. dublinensis*, *C. muytjensii*, *C. turicensis*, *C. universalis*, and *C. condiment*. The isolates of *C. sakazakii* have been suggested to be responsible for fatal neonatal infections, which gives rise to sepsis, necrotizing enterocolitis, and meningitis, with a high mortality rate. (1) In spite of highlighting neonatal infections caused by Cronobacter species, studies have indicated that these bacteria can induce illness in both infants and adults, particularly in newborns, the elderly, and those with weakened immune systems. (2)

The outbreaks of Cronobacter infections have recently been reported in many countries. (3–6) Due to the inappropriate and excessive use of antimicrobial agents, the emergence and spread of multidrug-resistant and extensive-drug resistant strains have globally become a matter of increasing concern for public health. (7) Current surveys have speculated that Cronobacter species is less resistance to widely used antibiotics in comparison to other newborn pathogens, though multiple investigations have found drug-resistant strains of Cronobacter species (8–10) Therefore, it is essential to explore the antibiotic resistance of Cronobacter species, particularly those that are recovered from different clinical samples, to categorize the patterns of resistance and to formulate an efficient strategy for inhibiting the potential spread of these strains.(11)

In recent years, a serious threat to newborn safety is the attachment and biofilm formation of newborn pathogens because the high likelihood of potential cross-contamination may result in serious problems for newborn safety. It has lately been found that the strains of Cronobacter species are capable of biofilm formation on numerous kinds of materials, e.g. stainless steel, polyvinyl chloride, silicone, and polycarbonate. The elimination of the established biofilms are highly difficult in virtue of the tolerance to sanitizing agents, posing a potential health risk to human health because within biofilms, there are microorganisms that may cause a persistent release of bacteria to newborn (12). The aim of the present study was to investigate the antimicrobial susceptibility and biofilm formation of *C. sakazakii* isolates from neonatal sepsis in Southwestern Iran.

Methods

Ethic statement

The present cross sectional study was conducted after obtaining approval from the Ethics Committee (IR.AJUMS.REC.1400.108) of the Ahvaz Jundishapur University of Medical Sciences and their submission to affiliated hospitals.

Study Design and Population

This study design was carried out cross-sectional study from March 2021 to March 2019. The study was conducted on all blood sample of neonatal sepsis sent to the microbiology lab of Imam Khomeini Hospital, Ahvaz, Iran. The hospital serves as a referral center for the public hospital in Ahvaz city. The study included 1045 preterm neonates of 60/min, signs of respiratory distress (marked chest in drawing), grunting, cyanosis, fever or hypothermia, and elevated C-reactive protein (CRP). For these neonates, blood cultures were collected and analyzed. Neonates were excluded from the analysis if the diagnosis of sepsis was presumptive and blood cultures were negative.

Data and Sample Collection

A total number of 1045 blood cultures were collected from cases suspected to neonatal sepsis admitted to the neonatal ICU at Imam Khomeini Hospital. The number of samples was limited by the consent rate. Neonates were subjected to complete clinical examination (temperature, respiration, color, presence of lethargy, or any neurological troubles and change in feeding pattern). Blood cultures were collected to be tested for the presence of bacterial infections, including *C. sakazakii*. Samples had been collected for general screening purposes. Approximately 1-4 ml of blood was aseptically collected from the peripheral vein and inoculated directly into BacT/ALERT blood culture bottles (bioMérieux, Marcy l'Etoile, France), which was monitored using the BacT/ALERT 3D instrument (bioMérieux, Marcy l'Etoile, France). The bottles were incubated at 37C° for 7 days, and positive specimens were inoculated onto Brilliance C.

sakazakii chromogenic agar (DFI, Oxoid, United Kingdom), MacConkey agar, and blood agar and incubated for 24 h at 36°C. Also, a sample was streaked onto trypticase soya agar, which was incubated at 25°C. (14) Suspected colonies were further confirmed using *C. sakazakii* specific primer confirmation.

DNA extraction

The boiling colonies of *C. sakazakii* strains grown overnight on nutrient agar (Merck, Germany) were suspended in microtubes containing 500 µL of Tris–HCl–EDTA buffer, then the microtubes were placed in thermoblock (Denville Scientific, Metuchen, NJ, USA) for 5 min at 95°C, and centrifuged at 14 000 rpm for 10 min at 4°C. The supernatant was used as the DNA template in the PCR assays. UV absorbance ratios, A_{280}/A_{260} were used to evaluate DNA extract purity using a Nanodrop instrument (Thermo Scientific, Waltham, MA, USA). method was used to extract genomic DNA from *C. sakazakii* isolates.

Confirmation of *C. sakazakii*-specific PCR amplification primers

For definitive identification, all 43 suspected *C. sakazakii* isolates were exposed to *C. sakazakii*-specific PCR amplification primers. The *C. sakazakii* (ATCC 51329) strain was used as a positive control and *Staphylococcus aureus* ATCC 25923 strain was the negative control.

Antibiotic resistance AMR profiles

The AMR of the bacterial isolates to antibiotics is determined by the disk diffusion method according to procedures of the CLSI (2021). The isolates are classified as sensitive and resistant based on the diameter of the clearing zone according to CLSI (2021) guidelines. The tested antibiotics included ampicillin (10 µg), amoxicillin (25 µg), ampicillin-sulbactam (20 µg), tetracycline (30 µg), gentamycin (10 µg), erythromycin (15 µg), clindamycin (2 µg), tobramycin (10 µg) amikacin (30 µg), chloramphenicol (30 µg), cefoxitin (30 µg), Cefepime (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), levofloxacin (10 µg), and imipenem (10 µg), meropenem(10 µg), doripenem(10 µg). The phenotype defined as multiple drug resistance (MDR), extremely drug resistant (XDR) and pandrug resistant (PDR) according to the International Expert proposal for Interim Standards Guidelines(15,16).

Minimum inhibitory concentration (MIC)

Colistin MICs were measured by E-test strips (Liofilchem, Italy) and interpreted based on (CLSI 2020) guidelines. After measuring the MIC with E-test method, the *P. aeruginosa* isolates with a MIC Equal to and lower than 2 µg/ml were considered as “intermediate” and MIC Equal to and higher than 4 µg/ml were considered as “resistant”. *Escherichia coli* ATCC 25,922 was used as quality control for antimicrobial susceptibility testing (15).

Biofilm formation

in 96-well microtiter plate (MTT) the biofilm formation capacity of isolates was evaluated using the crystal violet staining method. First, these isolates were inoculated in Mueller–Hinton agar at 37°C overnight. Then, these isolates were adjusted to 0.5 McFarland ($\sim 1.5 \times 10^8$ CFU/ mL) with normal saline (0.85% NaCl). A 10-µL aliquot of each suspension was then diluted 1:200 in 190 µL of tryptic soy broth (TSB) containing 1% glucose in 96-well polystyrene microtiter plates. Following incubation at 37°C overnight, the plates were washed three times with PBS, fixed by adding 200 µL of methanol into each well, and stained with 200 µL of 0.1% crystal violet (CV) for 20 minutes. The plates were again washed three times to remove excess stain, and the remaining CV was solubilized by incubating with 200 µL of 95% ethanol for 10 minutes. The optical density at 570 nm (OD₅₇₀) of each well was measured by the ELISA plate reader (µQuant; BioTek Instruments, Winooski, VT, USA), to evaluate the biofilm formation capacity. *S. epidermidis* ATCC 35984 and TSB broth were used as positive and negative controls (OD_c) for the biofilm formation, respectively. The results were interpreted according to the criteria suggested by Zhang et al. Briefly, the isolates were classified into the several groups about the biofilm formation capacity: $OD_{570} \leq OD_c$ =no biofilm producer; $OD_c < OD_{570} \leq 2 \times OD_c$ =weak biofilm producer; $2 \times OD_c < OD_{570} \leq 4 \times OD_c$ =moderate biofilm producer; and $4 \times OD_c < OD_{570}$ =strong biofilm producer. All experiments were performed in triplicate (17).

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences, version 22 (SPSS Inc., Chicago, IL, United States). Data are represented as the mean ± SD for continuous variables and as percentages for categorical variables.

Results

Dissemination of positive cultures/specimens

The demographic and clinical characteristics of the neonates are summarized in Table 1 and table 2.

Table1-Demographic and clinical data of the study population.

ID	Sex	weight	Sepsis	MTP	ARPs	Colistin	MDR	XDR
1	F	< 2500	Late-onset sepsis	weak	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	+	-	+
2	M	1500-2500(VLBW)	Early-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
3	M	1500-2500(VLBW)	Late-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
4	F	1500-2500(VLBW)	Early-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
5	F	1500-2500(VLBW)	Late-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
6	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
7	M	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
8	F	< 2500	Early-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
9	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	+	-	+
10	F	1500-2500(VLBW)	Early-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
11	M	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
12	F	1500-2500(VLBW)	Late-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
13	M	1500-2500(VLBW)	Early-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
14	F	< 2500	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	+	-	+
15	M	< 1000(ELBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
16	F	1500-2500(VLBW)	Late-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
17	F	< 2500	Early-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	+	-	+
18	M	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
19	F	1500-2500(VLBW)	Late-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
20	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FEP,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	+	-	+
21	M	< 2500	Early-onset sepsis	weak	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	-	-	+
22	F	< 1000(ELBW)	Early-onset	-	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIP,LVX,NOR,C	+	-	+

			sepsis						
23	F	1500-2500(VLBW)	Late-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR,C	-	-	+	
24	F	< 2500	Late-onset sepsis	weak	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	-	-	+	
25	M	< 1000(ELBW)	Late-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	-	-	+	
26	F	1500-2500(VLBW)	Early-onset sepsis	weak	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	+	-	+	
27	F	< 2500	Early-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	-	-	+	
28	M	< 2500	Late-onset sepsis	weak	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	-	-	+	
29	M	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	+	-	+	
30	F	< 1000(ELBW)	Early-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	+	-	+	
31	M	< 2500	Early-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	+	-	+	
32	F	1500-2500(VLBW)	Late-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	+	-	+	
	F	< 1000(ELBW)	Late-onset sepsis		AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	-	-	+	
33	M	< 1000(ELBW)	Late-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FOX,DOR,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	+	-	+	
34	M	< 1000(ELBW)	Late-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	-	-	+	
35	M	1500-2500(VLBW)	Early-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	+	-	+	
36	F	< 1000(ELBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	+	-	+	
37	M	1500-2500(VLBW)	Late-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	-	-	+	
38	F	< 2500	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,TOB,AMK,CIPLVX,NOR	+	-	+	
39	M	1500-2500(VLBW)	Early-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,AMK,CIPLVX,NOR	+	-	+	
40	F	< 2500	Late-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,AMK,CIPLVX,NOR	-	-	+	
41	M	1500-2500(VLBW)	Early-onset sepsis	moderate	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,AMK,CIPLVX,NOR	+	-	+	
42	M	< 1000(ELBW)	Late-onset sepsis	-	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,AMK,CIPLVX	-	-	+	
43	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,T,E,CLI,FOX,IMP,MEM,GN,AMK,CIPLVX	-	-	+	

44	M	< 1000(ELBW)	Early-onset sepsis	-	AMP,AMX,AMS,E,CLI,FOX,IMP,GN,AMK,CIP,LVX	-	-	+
45	F	< 2500	Late-onset sepsis	strong	AMP,AMX,AMS,E,CLI,FOX,IMP,GN,AMK,CIP,LVX	-	-	+
46	M	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,E,CLI,FOX,IMP,GN,AMK,CIP,LVX	-	-	+
47	M	< 1000(ELBW)	Late-onset sepsis	-	AMP,AMX,AMS,E,CLI,FOX,IMP,GN,AMK,CIP,LVX	-	-	+
48	F	< 1000(ELBW)	Late-onset sepsis	weak	AMP,AMX,AMS,E,CLI,FOX,IMP,GN,AMK,CIP,LVX	-	-	+
49	M	< 1000(ELBW)	Late-onset sepsis	-	AMP,AMX,AMS,E,CLI,FOX,IMP,GN,AMK,CIP,LVX	-	-	+
50	M	1500-2500(VLBW)	Late-onset sepsis	weak	AMP,AMX,AMS,E,CLI,FOX,IMP,GN,AMK,CIP,LVX	-	+	+
51	F	< 1000(ELBW)	Late-onset sepsis	-	AMP,AMX,AMS,E,CLI,FOX,GN,AMK,CIP,LVX	-	+	-
52	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,CLI,FOX,GN,AMK,CIP,LVX	-	+	-
53	M	1500-2500(VLBW)	Late-onset sepsis	-	AMP,AMX,AMS,CLI,FOX,GN,AMK,CIP,LVX	-	+	-
54	M	1500-2500(VLBW)	Early-onset sepsis	strong	AMP,AMX,AMS,CLI,FOX,GN,AMK,CIP,LVX	-	+	-
55	M	< 1000(ELBW)	Late-onset sepsis	weak	AMP,AMX,AMS,CLI,FOX,AMK,CIP,LVX	-	+	-
56	F	< 1000(ELBW)	Early-onset sepsis	-	AMP,AMX,AMS,CLI,FOX,AMK,CIP,LVX	-	+	-
57	M	1500-2500(VLBW)	Late-onset sepsis	-	AMP,AMX,AMS,CLI,FOX,AMK,CIP,LVX	-	+	-
58	F	< 2500	Early-onset sepsis	weak	AMP,AMX,AMS,FOX,AMK,CIP,LVX	-	+	-
59	M	< 2500	Late-onset sepsis	strong	AMP,AMX,AMS,FOX,CIP,LVX	-	+	-
60	M	1500-2500(VLBW)	Early-onset sepsis	-	AMP,AMX,AMS,FOX,LVX	-	+	-
61	F	1500-2500(VLBW)	Early-onset sepsis	weak	AMP,AMX,AMS,FOX,LVX	-	+	-
62	F	< 2500	Late-onset sepsis	-	AMP,AMX,AMS,FOX	-	+	-
63	F	< 2500	Early-onset sepsis	-	AMP,AMX,AMS,FOX	-	+	-
64	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS,FOX	-	+	-
65	F	< 2500	Late-onset sepsis	weak	AMP,AMX,AMS,FOX	-	+	-

66	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS	-	+	-
67	M	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMX,AMS	-	+	-
68	M	< 1000(ELBW)	Late-onset sepsis	strong	AMP,AMX,AMS	-	+	-
69	F	1500-2500(VLBW)	Late-onset sepsis	-	AMP,AMX,AMS	-	+	-
70	M	< 2500	Late-onset sepsis	strong	AMP,AMX,AMS	-	+	-
71	M	1500-2500(VLBW)	Late-onset sepsis	-	AMP,AMX,AMS	-	+	-
72	F	< 1000(ELBW)	Late-onset sepsis	strong	AMP,AMS	-	+	-
73	F	< 2500	Late-onset sepsis	-	AMP,AMS	-	+	-
74	F	< 2500	Late-onset sepsis	weak	AMP,AMS	-	+	-
75	F	< 1000(ELBW)	Late-onset sepsis	strong	AMP,AMS	-	+	-
76	F	< 2500	Early-onset sepsis	strong	AMP,AMS	-	+	-
77	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMS	-	+	-
78	M	< 1000(ELBW)	Early-onset sepsis	-	AMP,AMS	-	+	-
79	F	< 1000(ELBW)	Early-onset sepsis	strong	AMP,AMS	-	+	-
80	M	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMS	-	+	-
81	M	1500-2500(VLBW)	Early-onset sepsis	strong	AMP,AMS	-	+	-
82	F	< 1000(ELBW)	Early-onset sepsis	weak	AMP,AMS	-	+	-
83	M	1500-2500(VLBW)	Early-onset sepsis	strong	AMP,AMS	-	+	-
84	M	1500-2500(VLBW)	Late-onset sepsis	strong	AMP,AMS	-	+	-
85	F	1500-2500(VLBW)	Early-onset sepsis	weak	AMP,AMS	-	+	-
86	M	< 1000(ELBW)	Early-onset sepsis	weak	AMS	-	-	-
87	F	1500-2500(VLBW)	Early-onset sepsis	weak	AMS	-	-	-
88	F	< 2500	Late-	strong	AMS	-	-	-

			onset sepsis						
89	F	< 2500	Late-onset sepsis	weak	AMS		-	-	-
90	F	1500-2500(VLBW)	Early-onset sepsis	strong	AMS		-	-	-
91	F	1500-2500(VLBW)	Early-onset sepsis	weak	AMS		-	-	-
92	M	1500-2500(VLBW)	Late-onset sepsis	strong	AMS		-	-	-
93	F	< 2500	Late-onset sepsis	strong	AMS		-	-	-
94	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMS		-	-	-
95	F	1500-2500(VLBW)	Late-onset sepsis	weak	AMS		-	-	-
96	M	1500-2500(VLBW)	Early-onset sepsis	moderate	AMS		-	-	-
97	F	< 2500	Early-onset sepsis	-	AMS		-	-	-
98	F	1500-2500(VLBW)	Late-onset sepsis	-	AMS		-	-	-
99	M	< 2500	Late-onset sepsis	moderate	AMS		-	-	-
100	F	1500-2500(VLBW)	Late-onset sepsis	strong	AMS		-	-	-
101	M	1500-2500(VLBW)	Late-onset sepsis	-	-		-	-	-
102	M	< 2500	Late-onset sepsis	moderate	-		-	-	-
103	M	1500-2500(VLBW)	Early-onset sepsis	-	-		-	-	-
104	M	< 2500	Early-onset sepsis	weak	-		-	-	-
105	F	1500-2500(VLBW)	Early-onset sepsis	moderate	-		-	-	-
106	M	< 2500	Late-onset sepsis	strong	-		-	-	-
107	F	1500-2500(VLBW)	Late-onset sepsis	moderate	-		-	-	-
108	F	1500-2500(VLBW)	Early-onset sepsis	strong	-		-	-	-
109	F	< 1000(ELBW)	Early-onset sepsis	-	-		-	-	-
110	M	< 1000(ELBW)	Late-onset	moderate	-		-	-	-

sepsis								
111	M	1500-2500(VLBW)	Early-onset sepsis	strong	-	-	-	-
112	F	1500-2500(VLBW)	Early-onset sepsis	moderate	-	-	-	-
113	M	< 2500	Early-onset sepsis	moderate	-	-	-	-
114	F	1500-2500(VLBW)	Late-onset sepsis	strong	-	-	-	-
116	F	< 1000(ELBW)	Early-onset sepsis	-	-	-	-	-
117	F	< 1000(ELBW)	Early-onset sepsis	-	-	-	-	-
118	F	< 2500	Late-onset sepsis	-	-	-	-	-
119	F	< 2500	Late-onset sepsis	weak	-	-	-	-
120	F	1500-2500(VLBW)	Early-onset sepsis	strong	-	-	-	-

Table2-Demographic and clinical data of the study population.

Variable	Number of cases (%)
Male gender	51 (42%)
Birth weight (g)	
Median (range)	1,500 (1,000–2,500)
Residence	
Rural	17 (14%)
Urban	103 (85%)
Cesarian section	68 (56%)
Central line insertion	6 (50%)
Maternal risk factors	
Preeclampsia	49 (40%)
Premature rupture of membranes	64 (53%)
Fever	100 (83%)
Antepartum hemorrhage	33 (27%)
Clinical manifestations	
Poor oral intake	51 (42%)
Fever >37.8°C	14 (11%)
Hypothermia <36°C	52 (43%)
Jaundice	40 (33%)
Eye discharge	28 (23%)
Skin rash	31 (25%)
Respiratory distress	76 (63%)
Apnea	43 (35%)
Pneumonia	67 (55%)
Platelet count (<100 cells/ μ l)	50 (41%)
Elevated CRP	120 (100%)
Total leukocytic count (cells/ μ l)	100 (100%)
Mean \pm SD	22.97 \pm 11.38
Median (range)	23.85 (2.9–49.0)

During the period of study, 734/1045 bacterial positive growth samples were collected from patients. Overall, from 734 bacterial positive growth samples 120 isolates were *C. sakazakii* based on culture, biochemical tests and PCR amplification. The remaining 614 positive blood cultures revealed other bacterial agents. *Klebsiella pneumoniae* was the most common microorganism causing neonatal sepsis (32%), followed by *Acinetobacter baumannii* (23%), coagulase-negative staphylococci (25%), methicillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* was detected in 10% of the cases. Most of the isolates were obtained from female patients (n=69; 57%) and the rest was male (n=51; 42%). seventy-four (61%) neonates had primary sepsis and 46 (33%) had late sepsis. Regarding birth bodyweight, 27 (22%) neonate weighted below 1000 gr, 61 (50%) between 1500 and 2500, and 32 (26%) more than 2500 gr.

Antimicrobials resistant rates of *C. sakazakii* isolates from neonatal sepsis

The resistance rates of *C. sakazakii* isolates to antimicrobial agents are illustrated in Table 3. In case of *C. sakazakii* isolates, the highest resistance rates belonged to Ampicillin (n=85/120; 70%), followed Amoxicillin (n= 71/120; %59) and Ampicillin/sulbactam (n=100/120; 83%). However, *C. sakazakii* had low levels resistance to cefepime and tetracycline. These isolates were then evaluating for colistin resistance of the MICs by using the E-test. Only (n= 20/120; %16) isolates were confirmed as colistin-resistant.

Table 3 - Results of antimicrobial resistance tests by disk diffusion method

Antimicrobial category	Antimicrobial agent	resistant	susceptible
PENICILLINS	Ampicillin	85(70%)	35(29%)
	Amoxicillin	71(59%)	45(33%)
B-LACTAM COMBINATION AGENTS	Ampicillin/sulbactam	100(83%)	20(0.16%)
TETRACYCLINES	Tetracycline	35(29%)	85(70%)
MACROLIDES	Erythromycin	51(42%)	69(57%)
LINCOSAMIDES	Clindamycin	57(47%)	63(52%)
CEPHEMS	Cefepime	25(20%)	95(79%)
	Cefoxitin	65(54%)	55(45%)
CARBAPENEMS	Doripenem	33(27%)	77(64%)
	Imipenem	50(41%)	50(4%)
	Meropenem	43(35%)	77(64%)
AMINOGLYCOSIDES	Gentamycin	54(45%)	66(55%)
	Tobramycin	38(31%)	58(48%)
	Amikacin	58(48%)	62(51%)
FLUOROQUINOLONES	Ciprofloxacin	59(49%)	61(50%)
	Levofloxacin	61(50%)	59(49%)
	Norfloxacin	41(34%)	79(65%)
PHENICOLS	Chloramphenicol	23(53%)	97(80%)
LIPOPEPTIDES	Colistin	17(14%)	103(85%)

Ampicillin (AMP), amoxicillin (AMX), Ampicillin/ sulbactam (AMS), Cefepime (FEP), Doripenem (DOR), Imipenem (IPM), Meropenem (MEM), Tobramycin (TOB), Gentamicin (GN), Amikacin (AMK), Ciprofloxacin (CIP), Levofloxacin (LVX), Norfloxacin (NOR), Tetracycline(T), Erythromycin(E), Cefoxitin(FOX), Clindamycin(CLI), Chloramphenicol (C)

Prevalence of MDR and XDR in *C. sakazakii* isolates

The prevalence rate of MDR and XDR *C. sakazakii* isolates from neonatal sepsis was identified by reference and shown in the table 4. Out of total 120 *C. sakazakii* isolated, (n=65; 53%) bacterial strains were MDR, and (n=53 ;44%) strains were XDR. No PDR isolated was detected in this study.

Biofilm formation rates of *C. sakazakii* isolates

The results of biofilm formation using the MTP method are shown table 4. In total, of the 120 isolated bacteria, (n=85 ;70%) were biofilm producers, of which, (n=45 ;37%) produced strong biofilms, (n=19; 15%) produced moderate biofilms, (n=21 ;17%) were weak biofilm producers and (n=34; 28%) were not biofilm producers.

Discussion

C. sakazakii is recognized as an opportunistic pathogen important for neonatal health worldwide. Neonatal sepsis, a major causes of neonatal mortality, is described as blood infections and imposes a significant health burden in infants, i.e. very low birth-weight and preterm infants (18). For the first time in the present study, *C. sakazakii* infection is reported in cases of neonatal sepsis in Ahvaz, Southwestern Iran.

Among our 734 blood cultures, 120 cases (16%) were positive for *C. sakazakii*, representing a higher rate than that reported in the United States, which was virtually 1 in 100,000 infants and increased to about 1 in 11,000 infants of less than 1,500 g birth weight (19). Our study was further investigated by Alsonosi and associates who genotyped 51 Cronobacter strains from clinical isolates collected during six years. Their result indicated that *C. sakazakii* (65%) is the major detected species (20). Evidence has also been demonstrated that the susceptibility of preterm infants to infections are greater than any other age. A reason for such observation can be the trans-placental passage of antibodies peaks during the third trimester. Thus, it can be deduced that the majority of preterm infants have considerably declined humoral immune responses (21).

The higher rate of colonization in our study likely display the patterns of admission into the NICU because almost all neonates were admitted within 24 hours of birth. A case of *C. sakazakii* was regarded as hospital-acquired if it was isolated from an outpatient or an inpatient within 48 h of hospitalization; therefore, it appears that all the *C. sakazakii* isolates have been transferred from the hospital. However, some studies have examined prematurity and intubation as

factors related to *S. aureus* colonization in the NICU population, but few have assessed the effect of age on colonization risk. We observed that neonates aged 7–14 and 15–28 days remarkably increased risk for *C. sakazakii* colonization compared to younger neonates, though we did not find any study in this matter for comparison. In this study, we discovered an association between higher birthweight quartiles and the increased risk of *C. sakazakii* colonization, which contradicts previous results. Our study also identified caesarian section delivery as a risk factor for *C. sakazakii*. Multidrug-resistant and extensive drug-resistant *C. sakazakii*, as central nosocomial infections, were critical problems in NICU. In our country, Iran, antimicrobial agents are extensively been misapplied, and this misapplication has complicated the treatment of infections associated with MDR and XDR *C. sakazakii*. In our study, 70% of *C. sakazakii* were resistant to one or more antimicrobial agents and 53% and 44% were MDR and XDR, respectively. A high level of antimicrobial resistance among *C. sakazakii* isolates has been reported in previous studies from Iran (22, 23). The high-level β -lactam combination agents and penicillin resistance among *C. sakazakii* isolates have caused serious problems in healthcare settings worldwide (24). Similarly, ampicillin/sulbactam-resistant enterococci (83%) was detected with higher frequency than ampicillin resistance (70%) in our study. A higher frequency of aminoglycoside resistance was also identified in *C. sakazakii* isolates, an observation that supports previous reports (25). Moreover, our results are consistent with previous reports on the predominance of *C. sakazakii* with two or more antibiotic-resistant categories (26).

In the current study, 14% of *C. sakazakii* isolates were high-level colistin-resistant, which is in good agreement with some former surveys (27, 28). Microbial cells within biofilms have been exhibited to be 10–1000 times more resistant to antibiotics than the planktonic cells (29). Based on the results obtained in this study, 66% of *C. sakazakii* strains showed biofilm formation with variable degrees, and islets containing biofilm had higher antibiotic resistance (Fig. 1). In general, the results of the Pearson and chi-Square test showed no significant difference between MDR and biofilm formation variables ($\chi^2 = 6.708$, $df = 3$, $P = 0.082$). Cronobacter species have varied abilities to form biofilms. Earlier studies have emphasized that *C. sakazakii* can attach to enteral feeding tubes within only 2 h of exposure. *C. sakazakii* also was able to bind to different surfaces viz latex, polycarbonate, and silicon. In biofilms, *C. sakazakii* are protected by secreted extracellular polymeric substances that form a protective shield from desiccation tolerance and abiotic stresses. Another difficulty related to the Cronobacter ability to form biofilms is that it renders these microorganism more resistant to disinfection. The ability of Cronobacter species to attach to infant feeding equipment may make these surfaces reservoirs and sources of infection for the neonatal (30). It has been reported that there is a strong correlation between the presence of antibiotic resistance and the ability of an *C. sakazakii* isolate, in order to colonizes and persists in neonatal and produces biofilm in vitro. According to our results, 73% of enterococci indicated biofilm formation phenotype, which signifies the vital role of biofilm formation in NICU. Cronobacter infections reported previously mainly include infants, particularly premature neonates, with clinical manifestation of sepsis.

Taken together, the high rate of *C. sakazakii* in neonates was high in the NICU. Age (7–28 days), higher birthweight, and caesarian delivery were the most remarkable risk factors for *C. sakazakii*. The majority of *C. sakazakii* strains were hospital-associated, which is the indication of NICU admission patterns. Our findings suggest that the active surveillance of neonates for *C. sakazakii* is required to be considered as a part of strategies to detect importation and prevent transmission of *C. sakazakii* within the NICU.

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee (REC) of the Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1399.38).

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in the present published article and its supplementary information file.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The concept and the design of the study were developed by Moloudsadat Motahar, Nazanin Ahmadvhosravi and Hossein Meghdadi. The methodology was designed by Ahmad farajzade sheikh and Fariba abbasi. Data collection and the experimental works were carried out by Zahra dargahi and Melica moradi and Shahla Samei Fard. The formal analyses and interpretation of data were carried out by Fatemeh Jahangirimehr. The original draft was prepared by Aram Asareh Zadehan Dezfouli and reviewed by Reza Heydari. All the authors have read and approved the final version of manuscript for submission. The Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran financially supported this project.

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Tables

Table 4 is not available with this version.

Figures

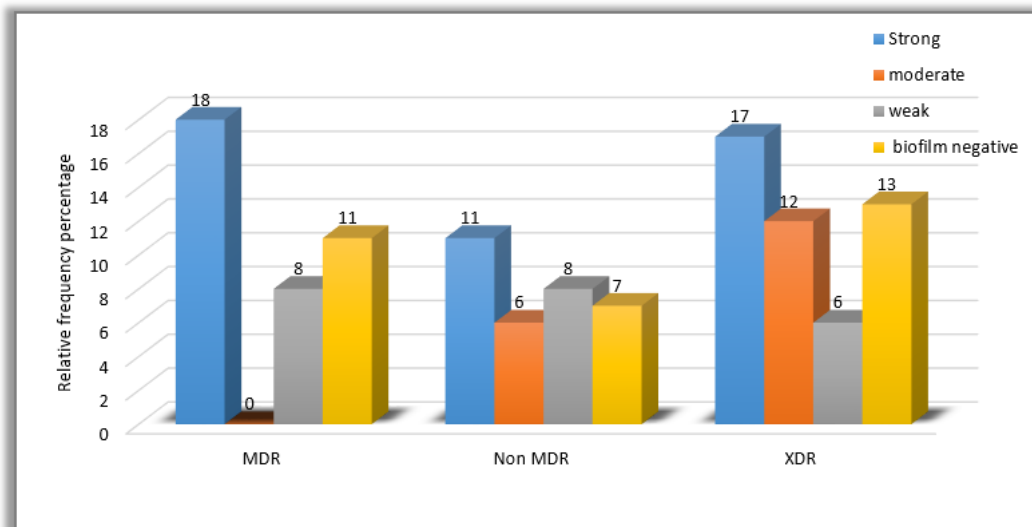


Figure 1

Determination of MDR, NonMDR and XDR biofilm capacity in *C. sakazakii* isolates