Letter to the Editor

Cronobacter Carriage in Neonate and Adult Intestinal Tracts

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A total of 7 *Cronobacter* strains were isolated from 703 fecal samples collected in Jinan from June 13 to December 30, 2011, with the positive rate of *Cronobacter* spp. being 1.0% (95% confidence interval 0.6%-1.4%). Three *Cronobacter sakazakii* stains were isolated from 157 fecal samples of healthy neonates (95% confidence interval 0.4%-5.5%). This number was slightly higher than that isolated from 273 fecal samples of healthy adults, in which 1 strain of *C. sakazakii* and 1 strain of *Cronobacter malonaticus* were isolated, and that from 173 fecal samples of adults with acute diarrhea, in which 1 strain of *C. sakazakii* and 1 strain of *C. malonaticus* were isolated, but the differences were not statistically significant (*P*>0.05). The *Cronobacter* isolates were all from different genetic sources. It should be noted that *Cronobacter* carriage may cause infection under certain conditions, especially in neonates.

Cronobacter spp. (*Enterobacter sakazakii*) are opportunistic pathogens which may cause lethal neonatal meningitis, septicemia, and necrotizing enterocolitis[1]. *Cronobacter* infections in adults are less severe, and generally affect patients with underlying disease[2]. Although *Cronobacter* spp. have been cultured from a broad range of human tissues and fluids[3], the *Cronobacter* carriage in human bodies and its relationship with infection are not fully understood. In an outbreak of *Cronobacter* spp. in France, a same type of *Cronobacter* strains was found to colonize intestinal tracts of some neonates without clinical symptom but infected other neonates simultaneously[4], and *Cronobacter* strains could attach to and invade human intestinal cells *in vitro*[5]. Therefore, intestinal pathway including attachment to intestinal cells and translocation to systemic tissue may play an important role in the pathogenesis of *Cronobacter*. *Cronobacter* strains colonizing neonatal intestinal tracts may infect hosts and others under certain conditions. So far, besides the report of *Cronobacter* spp. being isolated from a fecal sample and skin particles in adults[6], most documented human carriages of *Cronobacter*, especially in neonates, were detected in etiological survivors when *Cronobacter* infection outbreaks occurred[3-4,7]. In present studies, *Cronobacter* carriage in neonate and adult intestinal tracts was studied in the absence of *Cronobacter* infection outbreak.

A total of 703 fecal samples were collected to isolate *Cronobacter* spp. from June 13 to December 30, 2011, including 157 fecal samples of healthy neonates (≤1 week after birth) and 173 fecal samples of adults with acute diarrhea (≥18 years old) collected from the Fifth Hospital of Jinan, and 373 fecal samples of healthy adults (≥18 years old) collected from the physical examination clinic of Jinan Municipal Center for Disease Control and Prevention. "Healthy" was defined as without any clinical symptom 24 h before collection of the specimen for culture. "Acute diarrhea" was defined as having three or more loose stools 24 h before collection of the specimen for culture.

Fecal samples were collected by using Tanswab swabs (MW&E, Wiltshire, United Kingdom) containing Amies medium to reserve samples, and sent to laboratory for detection within 4 h. The swabs were aseptically cut into modified lauryl sulfate tryptose broth (mLST) / vancomycin medium (Landbridge, Beijing, China) which was incubated at 37 °C for 24 h. Then the cultures were streaked onto Druggan-Forsythe-Iversen (DFI) medium (Oxoid, Hampshire, United Kingdom). After incubating at 37 °C for 24 h, presumptive *Cronobacter* colonies (blue-green colonies) were picked and streaked onto tryptose soya agar (TSA, Landbridge) which was then cultured at 37 °C for 18 h. The colonies were identified by using Vitek 2 Compact system and Vitek GNI cards (bioMérieux, North Carolina, USA). Experimental procedure and quality control were carried out in accordance with the manufacturer's instructions.

DNA templates were extracted by using UNIQ-10 bacterial genomic DNA extraction kits (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. By using Taq DNA polymerase (Sangon), the *Cronobacter* isolates were amplified with 7f (5' CAG AGT TTG ATC CTG GCT 3') and 1492r (5' GGT TAC CTT GTT ACG ACT T 3'), or 50f (5' AAC ACA TGC AAG TCG AAC G 3') and 1492r. The following conditions were applied for an ABI 2720 thermal cycler (Applied Biosystems, California, USA): initial denaturation at 98 °C for 5 min; 35 cycles of denaturation at 95 °C for 35 s, annealing at 55 °C for

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35 s, extension at 72 °C for 90 s; and a final cycle at 72 °C for 8 min. The products were then sequenced by using a modified Sanger method with Big-dye Terminator v1.1 kits (Applied Biosystems) and an ABI 3730 capillary DNA analyzer (Applied Biosystems).

Additional sequences of reference strains were downloaded from the GenBank database including *C. sakazakii* ATCC 12868 [GenBank: EF059844], *C. sakazakii* ATCC 29004 [GenBank: EF059868], and ATCC 29544 [GenBank: EF059843]. 16S rRNA gene sequences (>1300 bases) were analyzed by using BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) with standard pairwise alignment method (open gap penalty of 100% and extend gap penalty of 0%). The alignments were verified by visual inspection, and similarity was calculated.

Multilocus sequence typing (MLST) of *Cronobacter* was determined by housekeeping genes: *atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB*, and *ppsA*. Premiers and protocol were available at web site: http://www.pubmlst.org/cronobacter/info/protocol.shtml. Seven digit allele codes, sequence types (STs), and species were obtained from *Cronobacter* MLST reference database (http://www.pubmlst.org/cronobacter).

Phenotypic tests were performed using conventional manual methods. They included motility, Voges-Proskauer, methyl red, indole, and ornithine decarboxylase tests. Reduction of nitrate to nitrite, production of gas from D-glucose, malonate utilization and production of acid from myo-inositol, dulcitol and melezitose were also determined.

Pulsed field gel electrophoresis (PFGE) of 7 *Cronobacter* isolates was performed according to the PulseNet USA protocol[8], by using the restriction enzyme *Xba* I. Gels were run at initial switch time of 2.16 s and final switch time of 63.8 s for 19 h at 6 V in a CHEF-MAPPER system (Bio-Rad, California, USA). PFGE types were analyzed by using BioNumerics software version 6.6 (Applied Maths). Patterns were compared and clustered by the unweighted-pair group method using arithmetic averages (UPGMA) by using Dice coefficient. Position tolerance was set to 1.5%, and optimization of 1.5% was applied during comparison of PFGE fingerprint patterns.

Antibiotic susceptibility test of *Cronobacter* isolates were conducted by using Vitek 2 Compact system and AST-GN13 cards (bioMérieux). Procedure and quality control were carried out according to the manufacturer's instructions.

Seven strains were isolated from 703 fecal samples and identified as *Cronobacter* spp. (99% probability). The total positive rate of *Cronobacter* spp. in samples was 1.0% (95% confidence interval 0.6%-1.4%), similar to the prevalence of *Cronobacter* spp. in human feces (1%, 95% confidence interval 0%-5.4%) and on human skin (0.9%, 95% confidence interval 0%-4.6%) reported by Kandhai et al.[6]. Table 1 shows the prevalence of *Cronobacter* spp. in different sampling groups. The positive rate in healthy neonates was slightly higher than those in healthy adults and adults with acute diarrhea, but the differences were not statistically significant (P>0.05). The positive rate of *Cronobacter* spp. in adults with acute diarrhea was not different from that in healthy adults (P>0.05), so there was no correlation between the *Cronobacter* carriage and the incidence of acute diarrhea, and this might be the normal colonizing intestinal tracks of *Cronobacter*. *Cronobacter* was detected in all three groups, indicating that healthy people usually can carry *Cronobacter*, and most carriage is harmless. The 3 neonates, who carried *Cronobacter* spp., were all delivered by caesarean section after full-term gestation (41 wk, 41 wk, and 39 wk, respectively) and with normal birth weight (3900 g, 4050 g, and 4150 g, respectively), they had breast-feeding and formula-feeding alternately. For a neonatal sample, the growing colonies were all blue-green, and five colonies randomly picked were all identified as *Cronobacter* spp. (99% probability). The 3 *Cronobacter* isolates from neonates were named as E1, E069, and E158. The 4 isolates from healthy adults and adults with acute diarrhea were named as D209, 254, C29, and C48 respectively. All the 7 *Cronobacter* isolates were from different genetic sources which might be related with foods, water, animals, plants, and environments indicated by the PFGE fingerprint patterns (Figure 1).

Table 1. Prevalence of *Cronobacter* spp. Isolated from Fecal Samples

Groups	Prevalence[a]	Positive Rate (%)	95% Confidence Interval (%)[b]
Healthy neonates	3/157	1.9	0.4-5.5
Healthy adults	2/373	0.5	0.1-1.9
Acute diarrheal adults	2/173	1.2	0.1-4.1

Note. [a]Positive/total. [b]The 95% confidence interval was calculated, assuming the drawing process is a series of independent draws from a binomial distribution with the probability P.

C48 and E069 were amplified with 7f and 1492r; 254, E1, C29, E158, and D209 were amplified with 50f and 1492r. Seven sequences of 16S rRNA gene of *Cronobacter* spp. were obtained and submitted to GenBank. The accession numbers were as follows: E1 [GenBank: JX035790], E069 [GenBank: JX035791], E158 [GenBank: JX035792], C29 [GenBank:

JX035787], C48 [GenBank: JX035788], D209 [GenBank: JX035789], 254 [GenBank: JX035786]. The sequence similarities between the 16S rRNA genes of the 7 *Cronobacter* isolates and ATCC 29544, ATCC 12866, and ATCC 29004 reference strains were calculated by using the BioNumerics programme. The sequences of the 7 isolates showed 99.3% to 99.9% similarity to the 3 reference strains. Therefore all the 7 isolates belonged to the group of *C. sakazakii* and *C. malonaticus*[9]. The 16S rRNA genes of isolates E1, E069, and 254 were more different from the reference strains. They had more mutation sites relatively than other four isolates in this study.

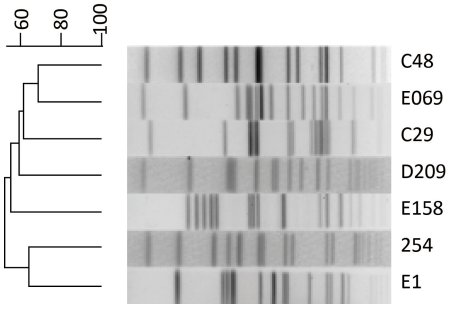

Figure 1. Dendrogram generated from PFGE profiles of *Cronobacter* isolates (*n*=7).

The specific phenotype results are shown in Table 2. E1, E069, E158, C48, and D209 were *C. sakazakii*; 254 and C29 were *C. malonaticus*. The species determined by biochemical tests were consistent with that determined by MLST. The STs of E1, E069, E158, C29, and C48 were ST156, ST157, ST158, ST161, and ST154 respectively; they were all new STs in the database, and clonal complexs were not defined. The ST of D209 was ST40, belonging to clonal complex 6. The ST of 254 was ST7, belonging to clonal complex 2 in which the clinical isolates were mainly from adults. The *C. sakazakii* isolates did not belong to clonal complex 4 which was a genetic signature for *C. sakazakii* neonatal meningitis[10], but other strains which are not from the clonal complex 4 have also been associated with neonatal meningitis, and no correlation has been found between specific STs and other clinical symptoms such as necrotizing enterocolitis[10]. Thus, although most *Cronobacter* carriage is harmless, the carriage of the opportunistic bacteria may cause infections under certain conditions, especially in those with underlying diseases.

For the 157 fecal samples collected from neonates, all the 3 isolates were *C. sakazakii*, and the *C. sakazakii* strain isolated from 1 neonate's fecal sample was the only bacterium growing on the DFI plate, so it might be the predominant bacterium in the neonate's intestinal tract. For the 373 healthy adults' fecal samples, one isolate was *C. sakazakii*, and another one was *C. malonaticus*, indicating that *C. sakazakii* is more likely to colonize neonate's intestinal track than adult's intestinal track. It might explain the fact that *Cronobacter* can cause more severe clinical complications in neonates than in adults[10]. Only adults were found to carry *C. malonaticus*, suggesting that *C. malonaticus* is more associated with adult infection than neonatal infection[10].

The sensitivities of E069, C48, C29, D209, 254 to cefazolin were intermediate, and the sensitivity of 254 to nitrofurantoin was also intermediate, but they were all sensitive to other antibiotics in this study. The sensitivities of all the isolates from adults to cefazolin were intermediate as well as one isolate from the neonate. It has been reported that some *Cronobacter* strains isolated from fatal cases produced extended-spectrum beta-lactamase[4]. Thus the *Cronobacter* colonizing human intestinal tracks also have the potential to develop resistance to cephalosporins.

Table 2. Assignment of Isolates to the Biogroups

Isolate Number	Biogroup	Phenotype[a]										
		VP	MR	Nit	Orn[b]	Mot	Ino	Dul	Ind	Malo	Gas	Mlz
E069, D209	1	+	-	+	+	+	+	-	-	-	+	-
E1, E158, C48	2	+	-	+	+	+	-	-	-	-	+	-
C29, 254	9	+	-	+	+	+	-	-	-	+	+	-

Note. [a]VP, Voges-Proskauer; MR, methyl red; Nit, nitrate reduction; Orn, ornithine utilization; Mot, motility at 37 °C; Ino, acid production from inositol; Dul, acid production from dulcitol; Ind, indole production; Malo, malonate utilization; Gas, gas production from glucose; Mlz, melezitose. [b]From commercial biochemical test kits (Vitek 2 GN, BioMérieux).

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