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Letter

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Cronobacter sakazakii Infection Induced Fatal Clinical Sequels Including Meningitis in Neonatal ICR Mice

Hyun-A Lee[1], Sunhwa Hong[1], Hyoseok Park[2], Hoikyung Kim[2]* and Okjin Kim[1,3]*

[1]*Center for Animal Resources Development, Wonkwang University, Iksan, Republic of Korea*
[2]*Division of Human Environmental Sciences, College of Life Science, Wonkwang University, Iksan, Republic of Korea*
[3]*Institute of Biotechnology, Wonkwang University, Iksan, Republic of Korea*

***Cronobacter sakazakii* (*C. sakazakii*), formerly *Enterobacter sakazakii,* is an emerging pathogen associated with the ingestion of contaminated reconstituted formula that causes serious illnesses such as bacteremia, septicemia, necrotizing enterocolitis, meningitis and death in low-birth-weight preterm neonatal infants. The objective of this study was to develop an animal model for human neonatal *C. sakazakii* infections. We acquired timed-pregnant ICR mice and allowed them to give birth naturally. On postnatal day 3.5, each pup was administered orally a total dose of approximately 10^7 CFU *C. sakazakii* strain 3439. Mice were observed twice daily for morbidity and mortality. At postnatal day 10.5, the remaining pups were euthanized, and brain, liver, and cecum were excised and analyzed for the presence of *C. sakazakii*. *C. sakazakii* was isolated from cecum and other tissues in inoculated mice. In the tissues of *C. sakazakii* infected mice, meningitis and gliosis were detected in brain. In this study, we confirmed the neonatal ICR mice may be used a very effective animal model for human neonatal *C. sakazakii* infections.**

Keywords: *Cronobacter sakazakii*, *Enterobacter sakazakii*, infant, animal model, ICR mouse

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Neonatal infections believed to have been caused by *Cronobacter sakazakii* (*C. sakazakii*), formerly *Enterobacter sakazakii* (Iversen *et al*, 2008), were first reported by Urmenyi and Franklin (1961). Cronobacter are motile peritrichous, Gram-negative, rod-shaped, non-spore-forming bacteria (Iversen *et al*, 2004). Cronobacter is recognized worldwide as an emerging foodborne pathogen (Drudy *et al*, 2006). Cronobacter is distributed and frequently contaminated in the environment, plant materials, powdered infant formulas, cereal foods, fermented beverages, fruits, and vegetables (Drudy *et al*, 2006). In particular, contamination on powdered infant formula occurs more easily because it is a nonsterilized product (Beuchat *et al*, 2009). *C. sakazakii* infections are an important cause of life-threatening meningitis, septicemia, and necrotizing enterocolitis in infants and neonates (Nazarowec-White and Farber, 1997; Lai, 2001; Drudy *et al*, 2006). Premature and low-birth-weight infants and those aged <28 days are considered to be more at risk than are older infants (Bar-Oz *et al*, 2001). To examine *C. sakazakii* infection, human studies are unethical because mortality is a possible outcome for suitable individuals (Richardson *et al*, 2009). Animal surrogate studies are essential for extrapolation of *C. sakazakii* infection in humans. The design of an animal model for *C. sakazakii* infection is fundamental in gaining knowledge of why premature and immunocompromised human infants are at greater risk of infection. Furthermore, an animal model will allow us to test different strains of *C. sakazakii* for virulence (Richardson *et al*, 2009). The objective of this study was to evaluate the usability of neonatal ICR mice as an animal model for human *C. sakazakii* infections.

We acquired specific pathogen-free (SPF) timed-pregnant ICR mice at gestation day 15 from Samtako (Osan, Korea). Dams were acclimatized and kept in an isolated SPF barrier

*Corresponding authors: Hoikyung Kim, Division of Human Environmental Sciences, College of Life Science, Wonkwang University, 344-2 Shinyoung-dong, Iksan, Jeonbuk 570-749, Republic of Korea
TEL: +82-63-850-6894
FAX: +82-63-850-6894
E-mail: hoikyung@wku.ac.kr
Okjin Kim, Center for Animal Resources Development, Wonkwang University, 344-2 Shinyoung-dong, Iksan, Jeonbuk 570-749, Republic of Korea
TEL: +82-63-850-6668
FAX: +82-63-850-7308
E-mail: kimoj@wku.ac.kr

room with regulated temperature (23±1°C), humidity (50±5%) and light/dark cycle (12/12 hours). The animals were fed sterilized pellet diet by 2 M rad radiation (Purina, Seongnam, Korea) and sterilized water *ad libitum*. Dams were allowed to give birth naturally. Litters averaged 11 pups were divided with 2 groups (infection and control) and kept in an opaque, polypropylene cage under a small animal isolator. Neonates were treated orally by gavage on postnatal day 3.5 by using a 24 gauge, 3.175 stainless steel animal feeding tube (Popper & Sons, New York, USA) attached to a 1 mL syringe. In the other study, the oral infectious dose has been approximated to range from 10^3 (Iversen and Forsythe, 2003) to $\geq 10^8$ CFU (Pagotto *et al*, 2003). In this study, 3.5 day-old mice pups (n=11) inoculated orally with 10^7 CFU of *C. sakazakii* strain 3439 (isolated from commercially manufactured powdered infant formula) in 0.1 mL of hydrated infant formula with feeding tube. Control mice (n=11) received saline through the same route. Mice were observed twice daily for morbidity and mortality. We defined morbidity as noticeable lethargy and change of skin color from pink to blue or grey. At postnatal day 10.5, the remaining pups were euthanized, and brain, liver, small intestine and cecum were excised and cultured for *C. sakazakii* from the neonates. Also, the tissues were submitted to histopathological analysis. All studies were performed in accordance with the Guide for Animal Experimentation by Wonkwang University and approved by the Institutional Animal Care and Use Committee of Wonkwang University. All efforts were made to minimize pain or discomfort of animals used.

Each organ was aseptically taken from each mouse, mixed with 100 mL of Enterobacteriaceae enrichment (EE) broth (BD/Difco, Sparks, USA), and pummeled in a stomacher (Seward Medical, London, UK) at 260 rpm for 1 min. The mixtures were incubated at 37°C overnight for enrichment of *C. sakazakii* in tissues. After incubation, the EE broth was streaked onto violet red bile glucose (VRBG) agar (BD/Difco) and incubated at 37°C for 16-18 hours. Cells from presumptive *C. sakazakii* colonies were streaked on tryptic soy agar (BD/Difco) and incubated at 25°C for 16-18 hours. Cells from yellow-pigmented colonies were subjected to confirmation using the API 20E kit. Simultaneously, 16s rRNA gene sequencing of cells from presumptive *C. sakazakii* colonies was performed by Macrogen (Seoul, Korea; http://www.macrogen.com). The 16s rRNA gene sequences were analyzed by an NCBI BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for identification of *C. sakazakii*.

For histopatholocal analysis, the exercised tissues were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Then, 4 μm sections were cut on microtome (Thermo Shandon, Cheshire, UK) and stained with hematoxylin and eosin.

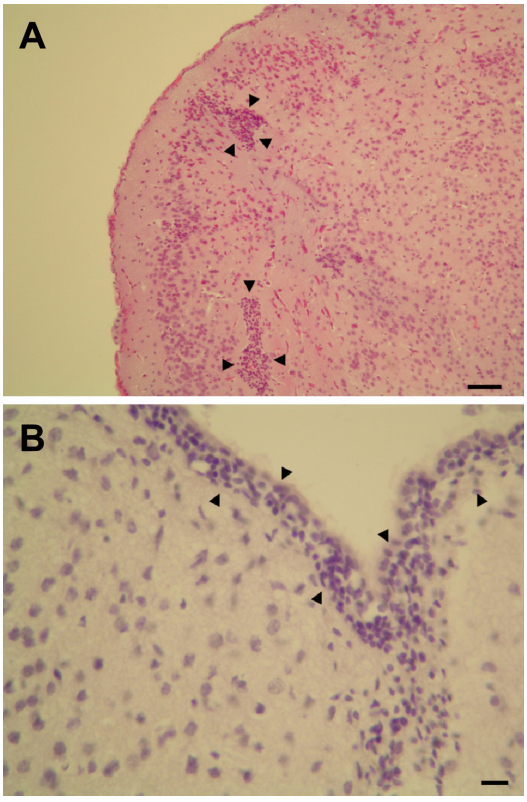

Figure 1. Histopathological findings of the cerebrum of *Cronobacter sakazakii*-infected mice. A: gliosis (black arrows), bar=100 μm. B: meningitis (black arrows), bar=50 μm. Hematoxylin-eosin stain.

In control mice, there were no dead animals during the experiment period. However, in mice of the infection group, 6 cases were dead at 3 days post-inoculation of *C. sakazakii*. Seven days after treating 3.5-day-old neonatal mice by oral gavage, *C. sakazakii* isolated from liver (3 cases in 5 survival pups), brain (2 cases in 5 survival pups), and cecum (5 cases in 5 survival pups) of *C. sakazakii* infected mice. In control mice, there were no histopathological changes. However, histopathological findings of the *C. sakazakii*-infected mice were meningitis and gliosis, and inflammatory cell infiltration in the cerebrum (3 cases in 5 survival pups) (Figure 1), and inflammatory cell infiltration and cellular degeneration in the liver (3 cases in 5 survival pups) (Figure 2), and inflammatory cell infiltration and cellular degeneration in the cecum (5 cases in 5 survival pups) (Figure 3).

C. sakazakii has been isolated from a wide range of environmental sources and foods of animal and plant origins, however, outbreaks of its infections have been linked only to powdered infant formula (Beuchat *et al*, 2009). Developing animal model as a surrogate for *C. sakazakii* infection in

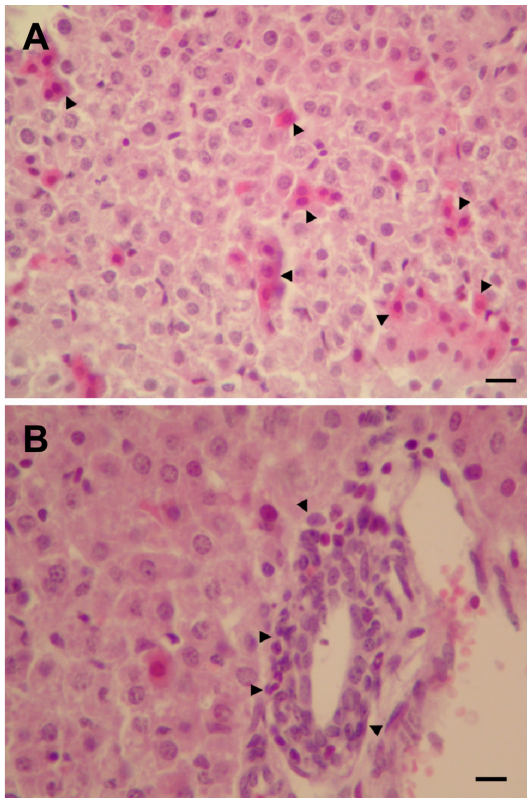

Figure 2. Histopathological findings of the liver of *Cronobacter sakazakii*-infected mice. A: hepatocytic degeneration (black arrows), bar=50 μm. B: inflammation (black arrows), bar=50 μm. Hematoxylin-eosin stain.

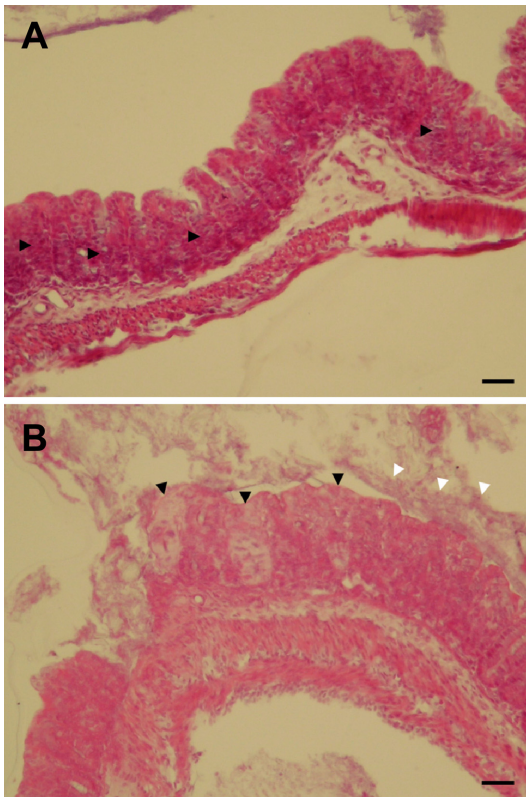

Figure 3. Histopathological findings of the cecum of *Cronobacter sakazakii*-infected mice. A: inflammation (black arrows), bar=100 μm. B: degeneration (black arrows) and bacterial colonies (white arrows), bar=100 μm. Hematoxylin-eosin stain.

premature infants is an important step forward, enabling subsequent research into understanding the mechanisms of infection, morbidity, prevention and treatment (Richardson *et al*, 2009). *C. sakazakii* is an opportunistic pathogen causing invasive infections (meningitis, sepsis, and necrotizing enterocolitis) with high death rates (40-80%), primarily in newborns (Bar-Oz *et al*, 2001; Iversen and Forsythe, 2003). In this study, we identified neonatal ICR mice as an animal model of *C. sakazakii* infection in premature infants. We observed *C. sakazakii* infection-related deaths in infected neonatal mice. Also, *C. sakazakii* isolated from liver, brain, and cecum of infected survival mice. In the *C. sakazakii*-infected mice, histopathological changes were detected in brain, liver and cecum. Our results indicate that *C. sakazakii* could be colonized and replication in ICR mice. Our results also show that *C. sakazakii* can induce meningitis and gliosis in neonatal mice. This is important because meningitis and other neurological squels are known to occur in human infants because of *C. sakazakii* infection. Mice are the most commonly used vertebrate species, popular because of their availability, size, low cost, ease of handling, and fast reproduction rate (Zak, 1999). ICR mice are the most popular strain of mice (Willis-Owen and Flint, 2006). In this study, we confirmed the neonatal ICR mice may be used a very effective animal model for human neonatal *C. sakazakii* infections. In conclusion, these results demonstrate that the neonatal ICR mice model may be used a very effective animal model for human neonatal C. *sakazakii* infections.

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