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Comparison of the pathogenicity among *Cronobacter* species in a neonatal mouse model

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Abstract

Neonatal infection caused by *Cronobacter* species can result in serious illnesses such as bacteremia, septicemia, meningitis, and death in at-risk infants who are orally fed contaminated reconstituted powdered infant formulas. The objective of this study was to compare the virulence among three *Cronobacter* species strains by using an animal model for human neonatal *Cronobacter* species infections. We acquired timed-pregnant ICR mice and all owed them to give birth naturally. On postnatal day 3, each pup was administered orally a total dose of 1×10^7 CFU *Cronobacter* species strain 3439, CDC 1123-79, and 3231. Mice were observed twice daily for morbidity and mortality. At postnatal day 10, the remaining pups were euthanized, and brain, liver, and cecum were excised and analyzed for the presence of *Cronobacter* species. *Cronobacter* species were isolated from cecum and other tissues in inoculated mice. In the tissues of *Cronobacter* species infected mice, meningitis and gliosis were detected in the brain. In this study, we identified the virulence among *Cronobacter* species strains by using a neonatal mice model which was a very effective animal model for human neonatal *Cronobacter* species infections.

Key words : *Cronobacter* species, Infant, Virulence, Animal model

INTRODUCTION

Cronobacter species were originally referred to as yellow pigmented *Enterobacter cloacae*, later being reclassified as a new species, *Enterobacter sakazakii* (Farmer et al, 1980). Later and following further extensive polyphasic analysis, Iversen et al (2008) proposed the reclassification of these bacteria into a new genus called *Cronobacter*.

Neonatal infections believed to have been caused by *Cronobacter* species, formerly *Enterobacter sakazakii* (Iversen et al, 2008), were first reported by Urmenyi and Franklin (1961). *Cronobacter* species are motile peritrichous, Gram-negative, rod-shaped, non-spore-forming bacteria (Iversen et al, 2004). *Cronobacter* species are recognized worldwide as an emerging foodborne pathogen (Drudy et al, 2006; Iversen and Forsythe, 2003). *Cronobacter* species are distributed and frequently contaminated in the environment, plant materials, powdered infant formulas, cereal foods, fermented beverages, fruits, and vegetables (Drudy et al, 2006; Iversen and Forsythe, 2003).

In particular, contamination on powdered infant formula occurs more easily because it is a nonsterilized product (Beuchat et al, 2009). *Cronobacter* species infections are an important cause of life-threatening meningitis, septicemia, and necrotizing enterocolitis in infants and neonates (Drudy et al, 2006; Lai, 2001;

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Nazarowec-White and Farber, 1997). 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 *Cronobacter* species 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 *Cronobacter* species infection in humans. The design of an animal model for *Cronobacter* species 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 *Cronobacter* species for virulence (Richardson et al, 2009). In previous study, the neonatal ICR mice were suggested a very effective animal model for human neonatal *Cronobacter spp.*, infections (Richardson et al, 2012).

The objective of this study was to compare the virulence between 3 strains by using a neonatal mice model as an animal model for human neonatal *Cronobacter* species infections.

MATERIALS AND METHODS

Animal inoculation & sample collection

We acquired specific pathogen-free timed-pregnant ICR mice at gestation day 15 from Samtasko (Osan, Korea). Dams were acclimatized and kept in an isolated SPF barrier 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, Korea) and sterilized water *ad libitum*. Dams were allowed to give birth naturally. Litters averaged 10 pups were divided with 4 groups (control and 3 infection groups with different strains) and kept in an opaque, polypropylene cage under a small animal isolator. Neonates were orally treated by gavage on postnatal day 3 by using a 24 gauge, 3.175 stainless steel animal feeding tube (Popper & Sons, Inc., USA) attached to a 1 ml syringe. Three different stranins of *Cronobacter* species, strain 3439, strain CDC 1123-79, and strain 3231, were obtained from Professor Hoikyung Kim' Labotaroty in Division of Human Environmental Sciences, Wonkwang University.

In this study, 3 day-old mice pups inoculated orally with 1×10^7 CFU of *Cronobacter* species strain 3439 (n=10), CDC 1123-79 (n=10), and 3231 (n=10) in 0.1 ml of hydrated infant formula with feeding tube. Control mice (n=10) 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, the remaining pups were euthanized, and the brain, the liver, and the cecum were excised and cultured for *Cronobacter* species from the neonates.

In addition, 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.

Bacterial isolation

Bacterial isolation was conducted following a previous reported method (Park et al, 2010). Briefly, each organ was aseptically taken from each mouse, mixed with 100 ml of Enterobacteriaceae enrichment (EE) broth (BD, USA), and pummeled in a stomacher (Seward Medical, UK) at 260 rpm for 1 min. The mixtures were incubated at 37°C overnight for enrichment of *Cronobacter* species in tissues. After incubation, the EE broth was streaked onto violet red bile glucose (VRBG) agar (BD, USA) and incubated at 37°C for 16～18 h. Cells from presumptive *Cronobacter* species colonies were streaked on tryptic soy agar (BD, USA) and incubated at 25°C for 16～18 h. Cells from yellow-pigmented colonies were subjected to confirmation using the API 20E kit. Simultaneously, 16s rRNA gene sequencing of cells from presumptive *Cronobacter* species colonies was performed by Macrogen Inc. (Seoul, Korea). The 16s rRNA gene sequences were analyzed by an NCBI BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for identification of *Cronobacter* species.

Histopathological analysis

Histological analysis was conducted following a previous reported method (Lee et al, 2011). Briefly, the dissected tissues were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Then, 4 um sections were cut on microtome (Shandon, UK) and stained with hematoxylin & eosin.

RESULTS

In control mice, there were no dead animals during the experiment period. However, in mice of the infection group, 6 cases were dead from 4 to 5 days post inoculation of *Cronobacter* species strain 3439. Also, in the *Cronobacter* species strain CDC 1123-79 infection and the *Cronobacter* species strain 3231 infection group, 8 and 7 cases were each dead from 3 to 5 days post inoculation. The mortality was 0, 60, 80 and 70% each in control, *Cronobacter* species strain 3439, CDC 1123-79 and strain 3231 group, respectively (Table 1). Seven days after inoculating 3 day oldneonatal mice by oral gavage, *Cronobacter* species isolated from the brain, liver, and cecum of *Cronobacter* species infected mice (Table 2). In mice of the *Cronobacter* species strain 3439 infection group, *Cronobacter* species was isolated in the brain (3 cases), the liver (1 case), and the cecum (4 cases), respectively. In case of the *Cronobacter* species strain CDC 1123-79 infection group, *Cronobacter* species was isolated in the brain (6 cases), the liver (6 cases), and the cecum (6 cases). Also, in mice of the *Cronobacter* species strain 3231 infection group, bacteria isolated in the brain (4 cases), the liver (4 cases), and the cecum (4 cases).

As the results of histopathological examination, in control mice, there were no histopathological changes. However, histopathological findings of the *Cronobacter* species infected mice revealed mild to moderate focal meningitis including neutrophils and monocytes and gliosis in the cerebrum. Also, mild focal inflammatory cell infiltration composed with neutrophils and monocytes was detected in the liver and the cecum. In mice of the *Cronobacter* species. strain 3439 infection group, the lesions were detected in the brain (4 cases), the liver (3 cases), and the cecum (5 cases). In case of the *Cronobacter* species. strain CDC 1123-79 infection group, the lesions were observed in the brain (7 cases), the liver (6 cases), and the cecum (6 cases). Also, in mice of the *Cronobacter* species. strain 3231 infection group, the lesions were observed in the brain (4 cases), the liver (6 cases), and the cecum (5 cases).

Table 1. Comparison of mortality induced by 3 different strains of *Cronobacter* species

Strain	No. of test	Death No.	Mortality (%)
3231	10	7	70
3439	10	6	60
CDC 1123-79	10	8	80
Control	10	0	0

DISCUSSION

Cronobacter species 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

Table 2. Isolation of *Cronobacter* species and histopathological lesions in tissues

Strain	n	Bacterial isolation			Lesion		
		Brain	Liver	Cecum	Brain*	Liver†	Cecum‡
3231	10	4	4	4	5	6	5
3439	10	3	1	4	4	3	5
CDC 1123-79	10	6	6	6	7	6	6
Control	10	0	0	0	0	0	0

*Brain showed mild to moderate focal meningitis and gliosis. †Liver revealed mild focal inflammatory cell infiltration composed with neutrophils and lymphocytes. ‡Cecum showed mild focal inflammatory cell infiltration composed with neutrophils and lymphocytes.

et al, 2009). Developing animal model as a surrogate for *Cronobacter* species infection in premature infants is an important step forward, enabling subsequent research into understanding the mechanisms of infection, morbidity, prevention and treatment (Richardson et al, 2009). *Cronobacter* species 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; Drudy et al, 2006).

In this study, we identified neonatal ICR mice as an animal model of *Cronobacter* species infection in premature infants. We observed *Cronobacter* species infection-related deaths in infected neonatal mice. Also, *Cronobacter* species isolated from liver, brain, and cecum of infected survival mice. In the *Cronobacter* species infected mice, histopathological changes were detected in brain, liver and cecum. Our results indicate that that *Cronobacter* species could be colonized and replication in ICR mice. Our results also show that *Cronobacter* species can induce meningitis and gliosis in neonatal mice. This is important because meningitis and other neurological squeals are known to occur in human infants because of *Cronobacter* species infection. Mice are the most commonly used vertebrate species, popular because of their availability, size, low cost, ease of handling, and fast reproduction rate (Ghiara et al, 1999). ICR mice are the most popular strain of mice (Willis-Owen and Flint, 2006). In this study, we identified the differences of mortality, isolation rate and histopathological lesions between the strains of *Cronobacter* species. Until now, there were no reports about comparison of the pathogenicity among *Cronobacter* species. Our results revealed that the *Cronobacter* species strain CDC 1123-79 was higher virulence than other 2 strains.

In this study, we identified the virulence between *Cronobacter* species strains by using a neonatal mice model which was a very effective animal model for human neonatal *Cronobacter* species infections.

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