

Mini Review

Preventing the Contamination of Powdered Infant Formula from *Cronobacter* Species and *Salmonella enterica*

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Abstract

Powdered infant formula (PIF) and dried milk products are not sterile using current manufacturing process, and can be contaminated by *Cronobacter* species, *Salmonella enterica*, and other pathogens during their production, storage, and reconstitution stages. *Cronobacter* species are emerging opportunistic pathogens that cause sepsis, meningitis, and necrotizing enterocolitis in infants, especially in premature, low-birth weight neonates, and those with other diseases or immuno-compromised individuals. Nontyphoid *Salmonella* are the most common foodborne pathogens which cause acute gastroenteritis. Some cases may progress to bacteremia, meningitis, and osteomyelitis, especially among young children and immuno-compromised individuals. Several outbreaks of *Cronobacter* and *Salmonella* infections among infants have been linked to the consumption of contaminated PIF or dried milk products. It is important to understand the epidemiological characteristics, the detection and identification methods, and the preventive strategies of these bacteria. This mini review will present an overview of the infections caused by *Cronobacter* species and *Salmonella enterica* that associate with PIF and dried milk products among infants and young children focusing on the routes of infections, outbreaks, detection methods, and preventive measures to understand the nature of the infections and to prevent the bacterial contamination.

INTRODUCTION

Powdered infant formula (PIF) and dried milk products are major and critical nutrient sources for babies if breastfeeding is impossible or insufficient. PIF and dried milk products are not sterile using current manufacturing process. PIF and dried milk products can be contaminated by *Cronobacter* (C) species, *Salmonella* (S) *enterica*, and other pathogens during their production, storage, and reconstitution stages. *Cronobacter spp.* and *Salmonella enterica* are listed as class A pathogens associated with PIF, and these bacteria are great threat to infant health [1]. *Cronobacter spp.* are Gram-negative, motile, facultative anaerobic bacilli, and they are emerging opportunistic pathogens that cause sepsis, meningitis, and necrotizing enterocolitis among infants (children <1 year old), especially among premature, low-birth-weight neonates (children <28 days old), and those with other diseases or immuno-compromised individuals [2]. *Cronobacter* also cause diarrhea, urinary tract infections, septicemia, pneumonia, and wound infection among children and adults. Infants are more likely to develop meningitis than other age

groups if they are infected with *Cronobacter*. Among 7 subspecies of *Cronobacter*, *C. sakazakii*, *C. malonaticus*, and *C. turicensis* are the most common subspecies isolated from infantile cases [3]. Although the incidence of *Cronobacter* infections is pretty low, the mortality of meningitis in infants is as high as 40-80% [4].

Salmonella enterica are Gram-negative, usually motile, facultative anaerobic and rod-shaped bacteria. Nontyphoid *Salmonella* (NTS) are the most common foodborne diseases causing self-limiting acute gastroenteritis. Bacteremia occurs in approximate 5-10% of patients, and some of the cases may progress to invasive infections such as meningitis and osteomyelitis, especially in young children and immuno-compromised persons. Several outbreaks of *Salmonella* infection among infants have been linked to the consumption of contaminated PIF and dried milk products [5].

This article will present an overview of the infections caused by *Cronobacter spp.* and *Salmonella enterica* that associate with PIF and dried milk products among infants and young children

focusing on the routes of infections, outbreaks, and preventive measures to understand the nature of the infection and to reduce the risk.

Infection caused by *Cronobacter spp.* and *Salmonella enterica* among infants

After ingestion via contaminated food such as PIF or dried milk products, *Cronobacter spp.* Or *Salmonella enterica* cause gastroenteritis. Some cases of *Cronobacter* infection may progress to bacteremia, meningitis, septicemia, necrotizing enterocolitis, and brain abscesses, and these infections may cause severe neurological impairments such as hydrocephalus, quadriplegia, and

Developmental delays [7,8]. In infants, the symptoms of *Cronobacter* infections often include fever, poor feeding, constant crying, sleepiness, and seizures. *Cronobacter* meningitis usually occurs in the first days or weeks on life. Typically, about 4-6 cases of *Cronobacter* infection are reported among infants annually in the United States. Premature infants and those with immuno-compromised individuals are at high risk for serious illnesses [1].

Cronobacter can also cause a variety of infections in digestive, respiratory, and urinary systems and in wounds among children and adults, especially in the elderly or people who have chronic or immuno-compromised diseases.

The common symptoms caused by NTS are fever, vomit, abdominal pain, and diarrhea, usually appear 6-12 hours after *Salmonella* infection, and may last up to 10 days [9]. Although gastroenteritis caused by NTS serovars usually is self-limiting, secondary bacteremia may occur in especially in young children and those with other diseases or immune-compromised individuals [10]. Bacteremia can result in septic shock and infections in brain, liver, spleen, gallbladder, and bone-marrow.

Incidence, outbreaks, and susceptible population

The first documented outbreak of neonatal meningitis due

to *C. sakazakii* occurred in England in 1958 [11,12]. After the outbreak, *Cronobacter* infections have been reported worldwide and have been found an increase among infants and children. The study of Muytjens et al. [13], analyzed the contamination rate in 141 PIFs from 35 countries. *C. sakazakii* and other members of *Enterobacteriaceae* were isolated from 52.5% PIFs made from 13 countries, and the bacterial concentration was less than 1 colony-forming unit (CFU)/g in the products. The data suggest that

PIF at low contamination level might cause problems for infant health. It has been reported that the incidence of *Cronobacter spp.* was 1 case per 100,000 infants under 1 year of age, and 8.7 per 100,000 infants among low-birth-weight infants, and 9.3 per 100,000 among very low-birth-weight infants in the United States [4,14]. Patrick et al. [15] investigated 544 cases of *Cronobacter* infection during 2003-2009 from 6 states of the United States. The incidence of *Cronobacter* infection was 0.66 per 100,000 populations of all ages, while the highest percentage of invasive infections was among children under 5 years of age [15]. In the Netherland, the meningitis cases infected with *Cronobacter* in all population was 0.5-0.7% [16]. Several outbreaks and cases caused by *Cronobacter spp.* are summary in Table 1. The incidence of *Salmonella* infections among infants was 121.6 cases per 100,000 infants in the United States, and it was approximately 8 times more than the incidence among other age groups [17].

Several *Salmonella* outbreaks have been documented to associate with consumption of PIF [5,6]. The most of these causative *Salmonella* strains are rare serovars such as Ealing, Tennessee, Anatum, and Agona. In an outbreak in 1985, 70 people were infected in the United Kingdom, all 48 infants among the cases have been fed with a dried milk product from one manufacturer, and S. Ealing was isolated from sealed packets of PIF [18]. Another outbreak (17 cases) occurred in the United Kingdom and France among infants between 1996-1997, and was confirmed to link to contaminated PIF by *S. anatum* [19]. Between 2004-2005 a *Salmonella* outbreak occurred among

Table 1: Several outbreaks and cases caused by *Cronobacter spp.* among infants.

Year	No. of cases	No. of mortality	Location Reference
1958	2 meningitis	2	England Urmenyi & Franklin, 1961 [11]
1977-1981	8 meningitis	6	The Netherland Muytjens et al. 1983 [53].
1988	2 bacteremia, 2 other diseases/colonization	0	Tennessee, US Simmons et al. 1989 [54].
1984	4 sepsis, 1 meningitis, 6 other diseases/colonization	4	Arseni et al. 1987 [55].
1986-1987	3 meningitis	1	Iceland Biering et al. 1989 [56].
1998	2 meningitis, 10 other diseases/ colonization	2	van Acker et al. 2001 [57].
2001	1 meningitis, 9 other diseases/ colonization	1	Tennessee, US CDC, 2002 [58]
2004	1 meningitis, 4 colonization	1	New Zealand Food safety, 2006 [59]
2004	5 infections, 5 colonization	2	France Coignard et al. 2004 [60].
2008	2 brain infections/infection	1	New Mexico, US CDC, 2009 [61]
2011	3 meningitis/bacteremia	2	Florida, Illinois CDC, 2012 [62]

Note: From 1986, in most of the outbreaks and cases *Cronobacter spp.* are confirmed to linked to Contaminated PIF [12,61-64].

infants in France [20], and 141 cases of infants were investigated, and the symptoms in most cases were water or bloody diarrhea and fever. Same pulsed-field gel electrophoresis pattern of *S. agona* was identified from the strains isolated from the clinical, PIF, and environmental samples [5,20].

Infectious routes

Cronobacter spp. have been isolated from a variety of food, food substances, food manufacturers, environments, and the digestive tracts of humans and animals [21-24]. PIF has been identified as one of important infectious sources, especially in neonatal care facilities. *Cronobacter* spp. has been detected from:

(1) dairy-based food including PIF and dried milk products. (2) plant-based food including cereals, wheat, rice, fruits, vegetables, legumes, herbs, and spices. (3) animal-based food such as meats, fish, and cheeses [4], water and soft drinks [21-24]. A study conducted by US Food and Drug Administration (FDA) in 2002 detected *Cronobacter* from approximated 23% of PIF samples [25]. Lee et al. also reported that *Cronobacter* were isolated from about 18.6% of food samples [22].

Except from food and environment, rodents and flies also serve as a route of contamination of *Cronobacter* spp. [1]. Hospital nurseries, household, and other environments are also contaminated sources of *Cronobacter* [26]. *Cronobacter* are isolated from the most of clinical samples such as blood, cerebrospinal fluid, bone marrow, urine, and feces. *Cronobacter* and *Salmonella* have been detected in PIF and dried milk products in dairy manufactures.

The contamination incidence of PIF was about 6.6% in filed surveys reported by FDA, and about 2.4-14% in international surveys [13,27-29].

INFECTIOUS DOSES

The infectious dose of *Cronobacter* by ingestion among infants was about 104 bacteria proposed by WHO/FAO in 2007 [1]. The infectious dose may decrease in premature neonates or those with low stomach acidity or immuno-compromised individuals.

Infectious dose varies among the *Salmonellae* serovars. For NTS, the infectious dose is approximately 103 bacteria by ingestion [30]. Infants, the elderly, or immune-compromised individuals may become infected with small infectious dose. Over-the-counter medicines such as anti-acid drugs or food like milk may lower the infectious dose by reducing stomach acidity.

PATHOGENESIS

Bacterial diseases of the gastrointestinal tract typically result from a complex set of interactions between the offending bacteria and the host. Although the mechanism of pathogenicity and the virulence factors of *Cronobacter* are not understood well, *Cronobacter* may adhere to, invade, transcytose across intestinal epithelial and endothelial cells, and survive in macrophages [31,32].

After ingestion of contaminated food or water, *Salmonella* adhere and invade to the intestinal cells by employing multiple virulence factor including flagella, fimbriae, a type 3 secreting system, and plasmids to survive and replicate, and to induce

intestinal infection and immune response [33].

Cronobacter spp. have been shown to be more resistant to heat, desiccation, acids, osmotic stresses, UV light, and antimicrobial reagents than other members of *Enterobacteriaceae*. The high resistance contributes to *Cronobacter* surviving in dried foods such as PIF and dried milk products, and the resistance may be attribute to their ability of adhering to hydrophilic and hydrophobic surfaces, forming biofilm, and expressing virulence factors [34-36]. *Cronobacter* can take the ingredients of PIF as potential nutrients to multiply and survive. These ingredients in PIF include lactose, whey and soy protein, vegetable oil, vitamin, minerals, corn syrup, and corn maltodextrin [27]. *Salmonella* can form biofilm too. The biofilm formed by cellulose, fimbriae, and other substances contributes to the ability of *salmonella* surviving in hash environment and antimicrobial reagents [37]. Host factors appear to play an important role in bacterial infections. Infants are more susceptible to infections of the foodborne pathogens such as *Cronobacter* and *Salmonella* than other age groups because their digestive and immune systems are not mature enough to fight against the offended bacterial pathogens, and infants produce less stomach acids or weaker stomach acidity than those of other age groups.

DETECTION AND IDENTIFICATION METHODS

Various techniques have been developed to detect and identify *Cronobacter* and *Salmonella* from PIF and dried milk products. Traditional methods for the isolation and identification of *Cronobacter* spp. require a nonselective pre-enrichment step followed by a selective *Enterobacteriaceae* enrichment (EE) culture overnight at 37°C. The EE broth is inoculated on Violet Red Bile Glucose Agar (VRBGA) and incubates overnight at 37°C. Presumptive colonies are selected and cultured on Trypti case Soy Agar (TSA) for about 3 days at 25°C, and yellow pigmented colonies are confirmed with a biochemical strip [38]. In the detection procedure of USFDA Bacteriological Analytical Manual (BAM) for *Cronobacter*, suspected PIF samples are incubated with buffered peptone water supplemented with antibiotics for about 20 h at 37°C. Following secondarily enrichment in buffered peptone water for 4-6 h, DNA is extracted and polymer chain reaction (PCR) or real-time PCR assays are used to confirm the target bacteria [39]. Culture-based methods for detection of *Salmonella* are a pre-enrichment culture in a non-selective buffered peptone water, subculture in two selective broths, and then inoculate to selected agar media (xylose-lysine-deoxycholate (XLD) agar, Hektoen enteric (HE), Brilliant Green Agar, or Bismuth Sulphite Agar) at 37°C for 24 hours [40]. A presumptive positive isolate is often analyzed by serotyping, phage typing, PFGE, and antibiotic susceptibility. Molecular methods for detection of NTS include PCR, qPCR, real-time PCR, matrix-associated laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS), biocensors, and others. The most popular genes for designing primers for PCR assays are *Salmonella* invasion gene *invA*, the flagellin gene *fliC*, the capsular gene *viaB*, and other virulence-related *hilA*, *sopB*, *stn*, *PefA*, *spvC* genes, and the 16s RNA [41,42].

A variety of *Salmonella* typing and subtyping methods have been developed. Phenotypic methods include serotyping and antimicrobial resistance (AMP) typing, whereas genotypic

methods include pulse-field gel electrophoresis (PFGE), ribotyping, and multilocus sequence typing (MLST).

Cronobacter identification and subtyping methods include PCR, ribotyping, gene sequencing, pulsed-field gel electrophoresis (PFGE), plasmid typing, and serogrouping assays [38]. Many virulence factors and housekeeping genes are chosen to design primers for PCR assays including 16S RNA, tRNA, *ompA*, *zpx*, *cpa*, *fhaB*, and others [38,43]. Multiplex PCR assay is a valuable tool to identify rapidly suspected bacteria. We designed primers targeting second messenger bis-(3'-5') - cyclic-dimeric-guanosine monophosphate (c-di-GMP) genes of *Cronobacter* spp. [44]. A *cgcA* gene, one of the c-di-GMP genes, presented in all *Cronobacter* species and was used to design several sets of primers to detect six *Cronobacter* species. In the multiplex PCR assay, 305 *Cronobacter* strains isolated from different sources including isolates from PIF samples were investigated. Our results demonstrate the multiplex method targeting *cgcA* gene is a highly sensitive and specific assay and can rapidly identify *Cronobacter* species in a single reaction [44].

PREVENTION

The strategies of preventing PIF from bacterial contamination should focus on improving the PIF production, storage, and reconstitution and keeping clean environments of neonatal health care facilities and household. Guidelines for PIF production, storage, and preparation have been instructed by the World Health Organization, USFDA, and CDC [45-48]. Controlling *Cronobacter* and *Salmonella* contamination is very difficult in a processing environment because of the widespread nature of the bacteria and the high resistance of the bacteria to heat, dryness, osmotic stress, UV light, and antimicrobial reagents [35,36]. Recent researchers based on international microbiological standards suggested that *Cronobacter* spp. should not be detected from 10 g of PIF [49]. Therefore, it is critical to imply reliable detection methods, effective environmental monitoring, and strict hygienic practices during PIF production, storage, and reconstitution to minimize neonatal exposure to the bacterial pathogens and to reduce the risk of severe infections.

Breastfeeding can prevent infants from the infections of these bacteria. For babies who depend on PIF or dried milk products, it is important to follow basic rules to avoid the multiplication of the pathogens in the products. The basic rules may include (1) choosing commercial sterile liquid infant formula for neonates, especially for premature neonates. (2) Preparing PIF carefully to achieve appropriate temperature, good personal hygiene, and enough mixing time. PIF should be consumed immediately after being reconstituted or stored under 5°C. (3) Washing the milk bottles and keeping clean working surfaces of countertops and sinks to minimize the entry of *Cronobacter* into the products and to remove any chances of bacterial multiplication [45,38].

Numerous methods and technologies are being developed to reduce/eliminate the potential bacterial contamination from PIF and dried milk products. These methods may include chemical (supercritical carbon dioxide, copper, lactic acids, and monolaurin), physical (gamma radiation, electron beam irradiation, heat, microwave, UV light, and near infrared

radiation), and biological methods (bioactive antimicrobial peptides, probiotics, and prebiotics) [50-52]. However, the methods are still in investigation or in evaluation for employing in PIF and dried milk products for their safety, flavor, quality, reproducibility and cost/benefit ratio issues [51,44].

CONCLUSION

PIF and dried milk products are not sterile. Several outbreaks of *Cronobacter* and *Salmonella* infections among infants have been linked to the consumption of contaminated PIF or dried milk products. PIF and dried milk products can be contaminated by the pathogenic bacteria during production, storage, and reconstitution stages. The prominent feature of the outbreaks of *Cronobacter* and *Salmonella* infections is that the bacteria are detected at low levels in PIF and dried milk products. The outbreaks and cases of the infections may represent only a small proportion of the actual incidences of *Cronobacter* and *Salmonella* infections among infants and young children. Thus, more sensitive and reliable detecting and screening methods should be developed to provide safe PIF and dried milk products. In every stage of production, storage, and reconstitution of PIF and dried milk products, it is critical to keep clean environments and strict hygiene standards to reduce/eliminate the bacterial contamination and multiplication.

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