Prevalence and characterization of *Salmonella enterica* in dried milk-related infant foods in Shaanxi, China

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ABSTRACT

The aim of this study was to investigate the existence and characteristics of *Salmonella enterica* in dried milk-related infant foods. Twenty-four (3.4%) of 705 samples, including 5 (2.0%) of 246 powdered infant formula, 18 (4.0%) of 445 infant rice cereal, and 1 (7.1%) of 14 other infant foods, were positive for *Salmonella*. Fifteen serotypes were identified in 40 *Salmonella* isolates; *Salmonella* Duesseldorf (15.0%) and *Salmonella* Indiana (15.0%) were more frequently detected than other serotypes. Resistance to chloramphenicol (82.5%) was most common, followed by tetracycline (57.5%), ceftiofur (52.5%), kanamycin (52.5%), streptomycin (50.0%), gentamycin (45.0%), nalidixic acid (35.0%), ceftriaxone (32.5%), ciprofloxacin (25.0%), amikacin (20.0%), and cefoxitin (15.0%). Twenty-eight (70.0%) isolates were resistant to ≥ 8 antimicrobials, with 5 (12.5%) being resistant to 14 antimicrobials. Amino acid substitutions in gyrase A (GyrA) were most frequently detected as Ser83Arg/Asp87Glu and in p53-associated Parkin-like cytoplasmic protein (ParC), they were all Ser80Arg; the quinolone resistance gene *qnrS* (47.5%) was commonly detected as well as aminoglycoside acetyltransferase [*aac(6')*-Ib; 25.0%], *qnrA* (17.5%), and *qnrB* (15.0%) genes. Thirty distinct pulsed-field gel electrophoresis patterns were identified among 40 isolates; no identical pulsed-field gel electrophoresis pattern was detected among *Salmonella* isolates with the same serovar that was recovered in 2010 and 2012. Our results suggest that dried milk-related infant foods could be contaminated with *Salmonella* and highlight that the dangers to infant health should not be neglected.

Key words: *Salmonella*, infant food, antimicrobial resistance, pulsed-field gel electrophoresis

INTRODUCTION

Dried infant foods, including powdered infant formula (PIF), infant rice cereal (IRC), and other infant foods (OIF) are not sterile products and may be intrinsically contaminated with pathogens, such as *Salmonella enterica* and *Cronobacter sakazakii*, which can result in serious illness among infants (i.e., children aged <1 yr; FAO/WHO, 2004). Although *C. sakazakii* has become an infant food safety issue due to the severity of disease it may cause in infants, concerns regarding *S. enterica* in milk-related infant foods have been somewhat overshadowed (Iversen and Forsythe, 2004; FAO/WHO, 2006).

Currently, the tolerance limits for *Salmonella* in milk and milk-related products in Canada, Cuba, Chile, European Union, the Netherlands, Australia, New Zealand, China, Singapore, and South Africa are all 0 cfu/25 g (mL) of sample, which means no *Salmonella* should be detected in milk, PIF, cheese, or yogurt (ICMSF, 1998; Wu et al., 2006). Although efforts have been made to control *Salmonella* in different milk-related products, in the 20-yr period from 1985 to 2005, at least 6 outbreaks of *Salmonella* infection have occurred in the United Kingdom, the United States, Canada, Spain, France, and Korea, associated with consumption of PIF, involving approximate 287 infants (Rowe et al., 1987; Centers for Disease Control and Prevention, 1993; Usera et al., 1996; Threlfall et al., 1998; Park et al., 2004; Brouard et al., 2007). The common characteristic of these outbreaks was the low level of *Salmonella* detected in the implicated PIF; thus, *Salmonella* may be ignored or missed during routine testing. The outbreaks likely represent only a tiny proportion of the actual number of *Salmonella* infections among infants that had been involved in PIF and OIF (Cahill et al., 2008).

In this study, the prevalence and characterization of *Salmonella* isolates recovered from dried milk-related infant foods were investigated in Shaanxi Province (China) to provide meaningful data for better understanding of infant food safety.

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MATERIALS AND METHODS

Sample Collection and Salmonella Isolation

A total of 705 dried milk-related infant foods, including 445 IRC, 246 PIF, and 14 OIF samples covering 71 brands, were randomly collected from 51 supermarkets and 32 retail stores in 12 cities (Xi'an, Yangling, Baoji, Meixian, Qishan, Qianxian, Xingping, Fufeng, Liquan, Zhouzhi, Wugong, and Xianyang) in Shaanxi Province (China) in 2010 and 2012. Two to 5 representative supermarkets and retail stores were selected in each city for sampling. In each supermarket/store, specific brands of IRC and PIF, including imported and domestically produced ones, were sampled.

Salmonella was isolated according to the standard procedures described in the National Standards of the People's Republic of China (GB 4789.4-2010). Briefly, 25-g food samples were placed in 225 mL of buffered peptone water (Beijing Land Bridge Technology Co. Ltd., Beijing, China) and incubated at $36 \pm 1^\circ\text{C}$ for 8 to 18 h. A 1-mL aliquot of preenriched culture was transferred to a 10-mL aliquot of tetrathionate (TT) broth (Beijing Land Bridge Technology Co. Ltd.) and selenite cystine (SC) broth (Beijing Land Bridge Technology Co. Ltd.). The TT was incubated at $42 \pm 1^\circ\text{C}$ and SC at $36 \pm 1^\circ\text{C}$ for 18 to 24 h. One loop of TT broth was streaked onto bismuth sulfite (BS; Beijing Land Bridge Technology Co. Ltd.) and SC broth onto Hektoen enteric (HE; Beijing Land Bridge Technology Co. Ltd.) agars. The BS plates were incubated at $36 \pm 1^\circ\text{C}$ for 40 to 48 h and HE plates for 18 to 24 h, and examined for the presence of *Salmonella*. The presumptive isolates on BS and HE plates were subcultured on Luria-Bertani agar plates (Beijing Land Bridge Technology Co. Ltd.) at $36 \pm 1^\circ\text{C}$ from 18 to 24 h, and 1 colony on Luria-Bertani plate was selected and confirmed with API20E identification kits (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions.

Serotyping

The O and H antigens of *Salmonella* isolates were characterized with hyperimmune sera (S&A Reagents Lab Ltd., Bangkok, Thailand; Statens Serum Institut, Copenhagen, Denmark). The serotype was identified using the slide agglutination method and assigned following the manufacturer's instructions and the Kauffmann-White classification scheme.

Antimicrobial Susceptibility Testing

Antimicrobial MIC were determined by an agar dilution method using Mueller-Hinton agar (Beijing Land

Bridge Technology Co. Ltd.) according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010). The antibiotics amikacin, gentamycin, kanamycin, streptomycin (STR), ampicillin, amoxicillin/clavulanic acid, ceftiofur, ceftriaxone, cefoxitin, trimethoprim-sulfamethoxazole, chloramphenicol, ciprofloxacin (CIP), nalidixic acid (NAL), and tetracycline were selected. *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *Enterococcus faecalis* ATCC 29212 were used as quality control organisms in MIC determinations. The breakpoints for interpretation of resistance and susceptibility were according to the interpretive standards of the Clinical and Laboratory Standard Institute (CLSI, 2010), except for STR, which was determined according to the breakpoints used for susceptibility testing of *Salmonella* and *Escherichia coli* of the National Antimicrobial Resistance Monitoring System (NARMS) of the United States (<http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm334828.htm>).

Screening for *qnr* and Aminoglycoside Acetyltransferase Genes, and Quinolone Resistance-Determination Region Gene Amplification and Sequencing

Salmonella isolates being resistant to NAL but susceptible or low-level resistant to CIP were examined for the presence of quinolone resistance (*qnr*) genes, including *qnrA*, *qnrB*, and *qnrS*, and the aminoglycoside acetyltransferase [*aac(6')*-Ib] gene. Isolates that showed both NAL and CIP resistance were examined for amino acid substitutions in quinolone resistance-determination regions (QRDR) of gyrase A (GyrA) and p53-associated Parkin-like cytoplasmic protein (ParC). All genes were amplified using MyCycler PCR (Bio-Rad Laboratories Inc., Hercules, CA) as described previously (Park et al., 2006; Cattoir et al., 2007; Yang et al., 2012). The primers were synthesized by TaKaRa Biotechnology Co. Ltd. (Dalian, China) and are listed in Table 1. The PCR products were stained with ethidium bromide and visualized under UV light after gel electrophoresis in 1% agarose. For DNA sequence analysis, PCR products were purified with a kit (TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0; TaKaRa Biotechnology Co. Ltd.), and sent for sequencing at AuGCT biotechnology Co. Ltd. (Beijing, China). The DNA sequence data were analyzed and aligned using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was carried out according to the protocol developed by the Centers

for Disease Control and Prevention (Ribot et al., 2006). Briefly, agarose-embedded DNA was digested with 50 U of the restriction enzyme *Xba*I (TaKaRa Biotechnology Co. Ltd.) for 1.5 to 2 h in a water bath at 37°C. The restriction fragments were separated by electrophoresis in 0.5 × TBE (Tris, borate, and EDTA) buffer at 14°C for 18 h using a Chef Mapper electrophoresis system (Bio-Rad Laboratories Inc.) with pulse times of 2.16 to 63.8 s. *Salmonella* Braenderup H9812 was used as the control strain. The gels were stained with ethidium bromide and visualized with UV transillumination (Bio-Rad Laboratories Inc.). The PFGE results were analyzed using BioNumerics software (version 3.0; Applied Maths, Kortrijk, Belgium) manually; the extent of variability was determined by use of the Dice coefficient and clustering was based on the unweighted pair group average method.

Statistical Analysis

The software DPS (DPS 9.5; Institute of Insect Science, Zhejiang University, Hangzhou, China) was used to process the prevalence, serovar distribution, antimicrobial susceptibility, and multidrug resistance of *Salmonella* isolates. Significant (*P* < 0.05) variation of positive rates among various foods (IRC, PIF, and OIF), serotypes, and antimicrobial susceptibility were determined using the Pearson chi-squared test at the 5% (with α = 0.05) level.

RESULTS

Prevalence of *Salmonella enterica*

Twenty-four (3.4%) of 705 dried milk-related infant food samples, including 5 (2.0%) of 246 PIF, 18 (4.0%) of 445 IRC, and 1 (7.1%) of 14 OIF, were positive for *Salmonella* (Table 2). A total of 40 *Salmonella* isolates of 15 serotypes were recovered. Among the isolates, *Salmonella* Duesseldorf (15.0%) and *Salmonella* Indiana (15.0%) were significantly (*P* < 0.05) more commonly detected than *Salmonella* Djugu (2.5%), *Salmonella* Mbandaka (2.5%), *Salmonella* Nola (2.5%), *Salmonella* Papuana (2.5%), *Salmonella* Poitiers (2.5%), *Salmonella* Remo (2.5%), and *Salmonella* Somone (2.5%). No significant difference (*P* > 0.05) was found among the prevalence of *Salmonella* Duesseldorf, *Salmonella* Indiana, and *Salmonella* Blockley, *Salmonella* Enteritidis, *Salmonella* Thompson, *Salmonella* Typhimurium, *Salmonella* Rissen, and *Salmonella* Kiambu (Table 3). *Salmonella* Duesseldorf was the dominant serovar in 2010; isolates with this serovar were mainly recovered from IRC, whereas *Salmonella* Enteritidis was the

Table 1. Primers used for detection and sequencing of target genes

Target gene	Primer ¹	Sequence (5'-3')	Annealing temperature (°C)	Product size (bp)	Reference
Amplification and sequencing primers	<i>gyrA</i> -F	ACGTACTAGGCAATGACTGG	56	190	Yang et al. (2012)
	<i>gyrA</i> -R	AGAAAGTCGGCGTCGATAGAA			
	<i>parC</i> -F	CTATGCGATGTCAGAGCTGG	54	270	Yang et al. (2012)
Detection primers	<i>parC</i> -R	TAAACAGCAGCTCGGCTATT	60	580	Cattoir et al. (2007)
	<i>qnrA</i> -F	AGAGGATTTCTCACGCCAGG			
	<i>qnrA</i> -R	TGCCAGGCACAGATCTTGAC	56	264	Cattoir et al. (2007)
	<i>qnrB</i> -F	GGMATHGAAATTCGCCACTG			
	<i>qnrB</i> -R	TTTGCGYGYGCCAGTCGAA	57	428	Cattoir et al. (2007)
	<i>qnrS</i> -F	GCAAAGTTCATTGAACAGGGT			
	<i>qnrS</i> -R	TCTAAACCGTCGAGTTCGGCG	55	482	Park et al. (2006)
<i>aac(6')-Ib</i>	<i>aac(6')-Ib</i> -F	TTGCGATGCTCTATGAGTGGCTA			
	<i>aac(6')-Ib</i> -R	CTCGAATGCCTGGCGTGTTT			

¹F = forward; R = reverse; *gyrA* = gyrase A; *parC* = p53-associated Parkin-like cytoplasmic protein; *qnr* = quinolone resistance; *aac(6')-Ib* = aminoglycoside acetyltransferase.

Table 2. Prevalence of *Salmonella* in powdered infant formula (PIF), infant rice cereal (IRC), and other infant foods (OIF)

Food	No. of samples	<i>Salmonella</i> -positive samples	
		No.	%
PIF	246	5	2.0
IRC	445	18	4.0
OIF	14	1	7.1
Total	705	24	3.4

dominant serovar in 2012 and mainly recovered from PIF (Figure 1).

Antimicrobial Susceptibility Testing

Antibiotic resistance was most frequently found to chloramphenicol (82.5%), the rate of which was significantly ($P < 0.05$) higher than those of tetracycline (57.5%), ceftiofur (52.5%), kanamycin (52.5%), STR (50.0%), gentamycin (45.0%), NAL (35.0%), ceftriaxone (32.5%), CIP (25.0%), amikacin (20.0%), and ceftiofur (15.0%). No significant ($P > 0.05$) difference was found among resistance rates of ampicillin (75.0%), amoxicillin/clavulanic acid (75.0%), and sulfamethoxazole/trimethoprim (65.0%; Table 4).

All isolates could resist at least 1 of the testing antimicrobials. Isolates that coresisted 8 testing antimicrobials could be found significantly more often ($P < 0.05$) compared with those coresistant to 5, 10, and 13 antimicrobials. No significant ($P > 0.05$) difference was found among the prevalence rates of isolates that

were coresistant to 1, 2, 5, 9, 10, 11, 12, 13, and 14 antimicrobials (Table 5).

Screening for *qnr* and Aminoglycoside Acetyltransferase Genes, and QRDR Gene Amplification and Sequencing

Twenty-one (52.5%) *Salmonella* isolates were positive for at least one of the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib* genes. Among these genes, *qnrS* (47.5%) was most frequently detected, followed by *aac(6')-Ib* (25.0%), *qnrA* (17.5%), and *qnrB* (15.0%). Eleven isolates (27.5%) were detected to carry at least 2 of the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib* genes, 8 (20.0%) carried at least 3 of the 4 genes, and 2 (5.0%) carried all 4 genes (Figure 1).

In 7 isolates, amino acid substitutions of QRDR in GyrA and ParC, and 1 of the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib* genes could be simultaneously detected. Mutations of Ser83Arg/Asp87Glu in GyrA were the most frequently detected; all mutations in ParC were detected as Ser80Arg.

PFGE

Thirty distinct PFGE based genotype patterns were identified, 21 were found in isolates recovered in 2010 and 9 in 2012. No identical PFGE pattern was found among *Salmonella* isolates of the same serovar that were recovered in 2010 and 2012 (Figure 1). Six *Salmonella* Duesseldorf isolates recovered from IRC of different brands in 2010 exhibited similar but different PFGE patterns as well as 5 *Salmonella* Enteritidis isolates. One *Salmonella* Indiana isolate recovered from baby cake showed a similar PFGE pattern to 5 other isolates recovered from IRC and PIF in 2010 and 2012.

DISCUSSION

Foodborne disease caused by nontyphoid *Salmonella* represents an important public health problem worldwide. Although data are not available in many countries, especially in developing countries, in the United States, the incidence of salmonellosis in infants was 121.6 laboratory-confirmed infections per 100,000 infants, which was approximately 8 times greater than the incidence in other age groups (Park et al., 2004; Cahill et al., 2008). Similarly high incidences of salmonellosis in infants have been reported as 181 cases per 100,000 infants in the United Kingdom and 92.8 cases per 100,000 infants in Israel (Skirrow, 1987; Weinberger et al., 2006). It is unclear if these *Salmonella* illnesses were associated with PIF and IRC; however, it is well known that PIF is the dominant food for the major-

Table 3. Distribution of *Salmonella* serotypes derived from powdered infant formula (PIF), infant rice cereal (IRC), and other infant foods (OIF)

<i>Salmonella</i> serotype	Isolates	
	No.	%
Duesseldorf	6	15.0 ^a
Indiana	6	15.0 ^a
Blockley	5	12.5 ^{ab}
Enteritidis	5	12.5 ^{ab}
Thompson	3	7.5 ^{ab}
Typhimurium	3	7.5 ^{ab}
Rissen	3	7.5 ^{ab}
Kiambu	2	5.0 ^{ab}
Djugu	1	2.5 ^b
Mbandaka	1	2.5 ^b
Nola	1	2.5 ^b
Papuana	1	2.5 ^b
Poitiers	1	2.5 ^b
Remo	1	2.5 ^b
Somone	1	2.5 ^b
Total	40	100

^{a,b}Values within a column followed by different superscript letters are significantly different ($P < 0.05$).

Figure 1. Pulsed-field gel electrophoresis (PFGE) pattern of *Salmonella* isolates recovered from infant rice cereal (IRC), baby cake (BC), and powdered infant formula (PIF). *qnr* = quinolone resistance gene; *aac(6')-Ib* = aminoglycoside acetyltransferase gene.

ity of infants, and IRC and OIF, such as baby cake, act as accessory infant foods that are not treated by sterilization. As a result, *Salmonella* may be intrinsically colonized in these foods. Although the Food and Agriculture Organization of the United Nations and the World Health Organization (WHO) categorized *Salmonella* as a category A hazard, and the tolerance limit for *Salmonella* in milk and milk-related products in most countries is 0 cfu/25 g (mL) of food, *Salmonella* in infant food has been responsible for infections and illnesses among infants (FAO/WHO, 2004; Forsythe, 2005). In addition to environmental contamination, PIF and IRC may become contaminated by *Salmonella* during preparation and handling in home and hospital settings, it is those preparations and handling that may introduce the hazards to PIF and IRC if hygienic practices are not followed (<http://www.who.int/foodsafety/publications/micro/mra10/en/>).

Compared with *Salmonella* serotypes among isolates recovered from retail chickens and meats, serotypes of isolates derived from milk-related infant foods were more diverse. These serotypes were unusual and not frequently detected; our results are consistent with the findings of the WHO (Chen et al., 2004; Yang et al., 2010; <http://www.who.int/foodsafety/publications/micro/mra10/en/>). According to the WHO reports, *Salmonella* of these rare serotypes associated with outbreaks could facilitate some outbreaks to be identified if the *Salmonella* surveillance network were well-established. Thus, if a serotype of *Salmonella* isolates that was recovered from milk-related infant foods during routine testing were identified the same as that in outbreaks, more attention should be paid to avoid illnesses among infants. Because we cannot share the PFGE data on *Salmonella* in the molecular subtyping network for foodborne bacterial disease surveillance (PulseNet)

Table 4. Antimicrobial resistance of *Salmonella* serovars derived from powdered infant formula (PIF) and infant rice cereal (IRC; n = 40)

Antimicrobial agent ¹	Resistant isolates	
	No.	%
CHL	33	82.5 ^a
AMPI	30	75.0 ^{ab}
A/C	30	75.0 ^{ab}
SXT	26	65.0 ^{abc}
TET	23	57.5 ^{bcd}
TIO	21	52.5 ^{cde}
KAN	21	52.5 ^{cde}
STR	20	50.0 ^{cde}
GEN	18	45.0 ^{cdef}
NAL	14	35.0 ^{defg}
CRO	13	32.5 ^{efgh}
CIP	10	25.0 ^{fgh}
AMK	8	20.0 ^{gh}
FOX	6	15.0 ^h

^{a-h}Values within a column followed by different superscript letters are significantly different ($P < 0.05$).

¹CHL = chloramphenicol; AMPI = ampicillin; A/C = amoxicillin/clavulanic acid; SXT = trimethoprim-sulfamethoxazole; TET = tetracycline; TIO = ceftiofur; KAN = kanamycin; STR = streptomycin; GEN = gentamycin; NAL = nalidixic acid; CRO = ceftriaxone; CIP = ciprofloxacin; AMK = amikacin; FOX = ceftiofur.

of China and the limited PFGE data we had, it is difficult for us to trace the source of *Salmonella* isolates recovered from PIF, IRC, and ORF.

Antimicrobial resistance in *Salmonella* has always been a significant public health concern worldwide (Su et al., 2004). Our results indicate that all *Salmonella* isolates were resistant to at least 1 of the antimicrobial agents tested, and almost 70.0% of the isolates were resistant to at least 8 antimicrobial agents, with 12.5% resistant to more than 14 antimicrobials. Because antimicrobial susceptibility data on *Salmonella* isolates derived from PIF and IRC have seldomly been reported, the extent of antibiotic resistance of the *Salmonella* isolates in current study was not clear. A recent study indicated that 31 CIP-resistant clinical *Salmonella*

isolates in China were also resistant to ≥ 8 other antimicrobial drugs, and 54% of human *Salmonella* isolates were multidrug resistant in Henan Province (Cui et al., 2008; Xia et al., 2009). On the other hand, *qnrA*, *qnrB*, *qnrS*, and *aac(6)-Ib* associated with NAL and fluoroquinolones were detected frequently in *Salmonella* isolates recovered from PIF and IRC in the current study; as *qnr* genes are commonly carried by plasmids, which can be transmitted to other antibiotic-sensitive pathogens through conjugation, the presence of *qnr* genes in *Salmonella* isolates are potential threats to consumers, especially to infants (Sjölund-Karlsson et al., 2009; Veldman et al., 2011).

CONCLUSIONS

Salmonella isolates were detected at a low prevalence rate in retail PIF and IRC in China; 15 serotypes were detected among the 40 isolates that showed multiple PFGE genotypes and multidrug resistance. Plasmid-associated *qnr* and *aac(6)-Ib* genes as well as amino acid substitution in GyrA and ParC were detected in quinolone- and fluoroquinolone-resistance isolates. Although it is not technologically feasible to reduce *Salmonella* contamination in sterile PIF and IRC, preventative measures such as hazard analysis critical control points (HACCP) should be taken to keep the levels of contamination of these products as low as possible.

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Table 5. Multidrug resistance of *Salmonella* serovars derived from powdered infant formula (PIF) and infant rice cereal (IRC; n = 40)

No. of antimicrobial agents	Isolates	
	No.	%
1	4	10.0 ^{ab}
2	6	15.0 ^{ab}
5	2	5.0 ^b
8	9	22.5 ^a
9	6	15.0 ^{ab}
10	2	5.0 ^b
11	4	10.0 ^{ab}
13	2	5.0 ^b
14	5	12.5 ^{ab}

^{a,b}Values within a column followed by different superscript letters are significantly different ($P < 0.05$).

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