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Full Length Research Paper

Antibiotic resistance pattern of *Klebsiella pneumoniae* and *Enterobacter sakazakii* isolates from powdered infant formula

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Morbidity and mortality of infant infections caused by contaminated powdered infant formula (PIF) have been reported worldwide, and pathogens like *Enterobacter sakazakii* and *Klebsiella pneumoniae* are important causative agents. To evaluate the prevalence of antibiotic resistance in *E. sakazakii* and *K. pneumoniae* that caused PIF contamination in Chinese market, all the isolates from PIF were analyzed for detecting resistance to antibiotics. 30 PIF samples were randomly purchased in Chinese market in 2009 and 7 *E. sakazakii* and 6 *K. pneumoniae* isolates were obtained from 8 samples (26.7%), the isolates were evaluated for antibiotics susceptibility by disk diffusion technique as recommended by the Clinical Laboratory Standards Institute (CLSI). Susceptibility results showed that each isolate had different levels of resistance to β-lactam antibiotics, while sensitive to fluoroquinolones and aminoglycosides. One *K. pneumoniae* and one *E. sakazakii* isolate almost resisted all Cephalosporins chosen; the double-disk synergy test (DDST) showed these two isolates producing extended spectrum β-lactamase (ESBL). This is the first report of ESBL-produing in *E. sakazakii* from powdered infant formula in China.

Key words: *Klebsiella pneumonia, Enterobacter sakazakii,* disk diffusion, antibiotic resistance, extended spectrum β-lactamase (ESBL).

INTRODUCTION

Recently, considerable attention has been directed at the microbiological safety of PIF (FAO/WHO, 2004; FAO/WHO, 2006). This has primarily been due to neonatal infections by *Enterobacteriaceae* include *E. sakazakii* and *K. Pneumoniae* which were associated with contaminated PIF (Caubilla-Barron et al., 2007; Forsythe et al., 2005). These products are not sterile, but are expected to comply with international microbiological standards.

Klebsiella pneumonia has been associated with various ailments such as urinary tract infection, septicemia, respiratory tract infection, diarrhea and other diseases (Podschum and Ullmann, 1988). An increased resistance to antibiotics has been reported in *K. Pneumoniae* as the widespread use of the third generation cephalosporins, β-lactam and broad-spectrum antibiotics (Chaudhary and Aggarwal, 2004; Kamatchi et al., 2009). Resistance of *Klebsiella* spp. to the cephalosporins such as oxyimino β-lactams was first described in 1980 and since then a steady increase in resistance against cephalosporins has been seen. The mechanism of resistance in *K. Pneumoniae to* cephalosporins is mediated by extended spectrum β-lactamases (ESBLs) ESBLs are encoded by transferable conjugative plasmids which often encode resistant determinants to other classes of antibiotics (Bonnet, 2004). Outbreaks of ESBL-producing *K. pneumoniae* infections have increased worldwide (Bradford, 2001). The plasmid-mediated resistance against cephalosporins can be spread among related and unrelated gram negative bacteria. Recent reports have highlighted the emergence of ESBL producing strains endowed with an extremely wide spectrum of antibiotic

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resistance, including resistance to Trimethoprim, Amikacin, Streptomycin and Gentamicin (Iroha et al., 2008).

E. sakazakii is an emerging formula-borne pathogen associated with the ingestion of contaminated powdered infant formula(PIF) that causes necrotizing enterocolitis, sepsis, and meningitis in low-birth-weight preterm neonatal infants (Drudy et al., 2006; Lai, 2001; Forsythe, 2005). *E. sakazakii* was formerly referred to as "yellow-pigmented" *Enterobacter cloacae*, and was characterized as a unique species almost 30 years ago (Muytjens et al., 1983). Most of the attention to *E. sakazakii*-related contamination of food products has focused on PIF. In 2002, the U.S. Food and Drug Administration (FDA) published a warning regarding the presence of ES in baby formula (FDA, 2002). PIF is not manufactured as a sterile preparation, and some heat-resistant *E. sakazakii* isolates expressed a higher level of *infB* (which encodes a translation initiation factor), than did the heat-sensitive isolates (Asakura et al., 2007). *E. sakazakii* may exhibit longterm persistence in dried infant formula and has been reported to be the only organism isolated after a 2.5-year period of storage (Riedel and Lehner, 2007).

While a reservoir for *E. sakazakii* is unknown, a growing number of reports suggest a role for powdered infant formula as a vehicle for infection (van Acker et al., 2001; Biering et al., 1989). Mortality due to infections from *E. sakazakii* was higher than 50%, in recent years it has decreased, even though it is still quite high (Fiore et al., 2008). More research is needed to determine virulence factors, genetic diversity, appropriate recovery and subspeciation methods, environmental niches, and methods for control of this emerging pathogen in foods and the environment.

MATERIALS AND METHODS

A total of 30 PIF samples were randomly purchased from the retail market in Nanchang, China. 13 Enterobacteriaceaes were obtained from 8 samples (26.7%), for identification, various biochemical feasibility tests were conducted by API 20E test kit and other traditional methods recommended by FDA. All isolates were subjected to susceptibility testing using Kirby and Bauer method of determining antimicrobial susceptibility and ESBL production was phenotypically determined using double disc synergy test.

Antibiotic susceptibility test

The antibiotic susceptibility was determined by Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates as recommended by the Clinical Laboratory Standards Institute (CLSI). The disks containing the following antibiotics (μg/disk) were used: Aampicillin(10), Carbenicillin(100), Piperacillin(100), Oxacillin(1), Cefazolin(30), Cephalothin(30), Cephradine(30), Cephalexin(30), Cefuroxime(30), Cefamandole(30), Cefatriaxone(30), Ceftazidime(30), Cefoperazone(75), Ciprofloxacin(5), Ofloxacin(5), Norfloxacin(10), Kanamycin(30), Amikacin(30), Neomycin(30). The diameter of the zone of inhibition for each antibiotic was measured and interpreted as resistant, intermediate susceptible or susceptible according to CLSI criteria.

ESBL screening and confirmation by phenotypic methods

The isolates showing reduced susceptibility to ceftazidime or cefotaxime or both were tested for ESBL production by double-disk synergy test (DDST) using four disks (μg): Cefotaxime (CTX) (30), Cefotaxime + Clavulanic acid (10), Cefatzidime (CAZ) (30), and Ceftazidime + Clavulanic acid (10). The inoculum and incubation conditions were the same as for standard disk diffusion recommendations. A≥5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was designated as ESBL-positive. *Escherichia coli* ATCC 25922 was used as the quality control strain.

RESULTS

Antibiotic susceptibility

A total of 13 Enterobacteriaceae isolates were obtained from 8 samples (26.7%) in Nanchang, 6 isolates of *K. pneumoniae* and 7 isolates of *E. sakazakii* were isolated from the samples in the laboratory. All the isolates demonstrated resistance to β-lactams with different level, while sensitive to Fluoroquinolones and Aminoglycosides (Table 1). The antibiotic resistances patterns of *K. pneumoniae* and *E. sakazakii* isolates to β-lactams are shown in Table 2, 100% of *K. pneumoniae* isolates were resistant to Oxacillin and Cephalexin, and 100% of *E. sakazakii* resistant to Oxacillin.

Multi-drug resistance

Through statistical analysis, we found the antibiotic susceptibility results showed that the isolates with different levels of multi-drug resistance (Table 3), isolates with resistance to 6 antibiotics or more accounted for 46.7%, in which isolates of *K. pneumoniae* accounted for 71.4%. The results indicated that multi-drug resistance is prevalent in isolates from PIF, which constitutes a huge threat on food safety, especially on the health of neonate.

Frequency of ESBL production

Of the 13 isolates tested, only 2 isolates (15.4%) indicated intense resistance to Cephalosporins. As the DDST showed the inhibitory zones of cefotaxime and ceftazidime were increased in the presence of clavulanic acid by more than 5 mm in 2 isolates, which confirmed ESBL production, they were resistant to the third-generation cephalosporins (3GC) tested as shown in Table 4.

DISCUSSION

Our study indicated that the contamination caused by *E. sakazakii* and *K. pneumoniae* poses a potential threat on infants in China. An increasing resistance to antibiotics

Table 1. Antibiotic susceptibility profiles of 13 isolates by disc diffusion method.

Antibiotics	Resistant No. of isolates (%)	Intermediate No. of isolates (%)	Sensitive No. of isolates (%)
AMP	7(53.8)	1(7.7)	5(38.5)
CAR	8(61.5)	1(7.7)	4(30.8)
PIPC	1(7.7)	1(7.7)	11(84.6)
OXA	13(100)	0	0
CFZ	2(15.4)	0	11(84.6)
CEP	8(61.5)	4(30.8)	1(7.7)
CH	3(23.1)	1(7.7)	9(69.2)
CEL	11(84.6)	1(7.7)	1(7.7)
CXM	3(23.0)	5(38.5)	5(38.5)
CFM	2(15.4)	0	11(84.6)
CRO	2(15.4)	0	11(84.6)
CAZ	2(15.4)	0	11(84.6)
CFP	2(15.4)	0	11(84.6)
CIP	0	0	13(100)
OFX	0	0	13(100)
NOR	0	0	13(100)
GEN	0	0	13(100)
KAN	0	0	13(100)
AMI	0	0	13(100)
NM	0	0	13(100)

Table 2. Outcome of resistance of *E. sakazakii* and *K. pneumoniae* isolates from PIF to β-lactam antibiotics.

Antibiotics	Bacterial isolates resistant to antibiotics (%)	
	***E. sakazakii* (n=7)**	***K. pneumoniae* (n=6)**
AMP	28.6	83.3
CAR	42.9	83.3
PIPC	14.3	16.7
OXA	100	100
CFZ	14.3	16.7
CEP	71.4	50
CH	28.6	33.3
CEL	42.9	100
CXM	71.4	50
CFM	28.6	16.7
CRO	14.3	16.7
CAZ	14.3	16.7
CFP	14.3	16.7

has been reported as the antibiotics were used for curing infections in the hospital, especially in the intensive care units (Kollef and Fraser, 2001).

Reports from 1960-1999 of antibiotic susceptibility of *E. sakazakii* indicate the organism is typically susceptible to ampicillin, tetracycline, chloramphenicol, gentamicin, and the third-generation cephalosporins. Stock and Wiedemann studied the specific antibiotic profiles of various *E. sakazakii* strains. Interestingly, no natural resistance to cephalosporins was detected in wild-type populations of *E. sakazakii*, and these strains appear to lack β-lactamases (Stock and Wiedemann, 2002). Our present study demonstrated that one of *E. sakazakii* isolates from PIF produced ESBL, which has never been

Table 3. Multi-drug resistance in isolates from PIF.

No. of antibiotics	Bacterial isolates resistant to antibiotics (%)	
	***E. sakazakii* (n=7)**	***K. pneumoniae* (n=6)**
<5	71.4	16.7
6 - 9	14.3	66.7
>10	14.3	16.7

Table 4. ESBL detection results of two isolates by DDST.

Species	Strain	3GC			DDST[a]				ESBL
		CRO	CAZ	CFP	20	25	30	CTX[b]	
E. sakazakii	90830	R	R	R	+	+	+	+	+
K. pneumoniae	90923	R	R	R	+	+	-	+	+

[a]Cefotaxime discs (30 µg) were placed adjacent to an Ceftazidime + Clavulanic acid disc (30-10 µg) at the indicated inter-disc distances(center to center) of 20, 25 and 30 mm.
[b]Cefotaxime (30 µg) and Cefotaxime + Clavulanic acid (30-10 µg) discs were used.
DDST double-disc synergy test; -, negative; +, positive.

reported in other studies in China.

E. sakazakii is considered a ubiquitous microorganism: it has been in fact isolated from a great variety of sites (Iversen and Forsythe, 2003): foods, water and several areas, including houses and hospitals. A recent article reported the presence of *E. sakazakii* in a lot of maternal milk stored in a milk bank (Fiore et al., 2008). In recent years, the International Commission on Microbiological Specifications for Foods has ranked *E. sakazakii* a "severe hazard for restricted populations (Drudy et al., 2006)." Powered infant formula is basically a non-sterile product, good for microorganisms to reproduce, easily been subject to bacterial contamination. Some Reports showed that *E. sakazakii* was thermotolerant, which made PIF products easily subject to its contamination. In the past few years, mortality due to *E. sakazakii* was higher than 50%. However, the pathogenesis of infection by this organism is not well elucidated (Ray et al., 2007).

During the past decades, ESBL-producing *K. pneumoniae* have emerged as one of the major multi-drug resistant organisms (Vercauteren et al., 1997). Most ESBL-producing *K. pneumoniae* were isolated from hospital and clinical environment, it has become an important clinical problem due to their resistance to multiple antibiotics (Kim et al., 2002). The incidence of ESBL producing strains among clinical *Klebsiella* isolates has steadily increased over the past few years. Since ESBL are most often encoded on plasmids and these plasmids also encode other antibiotic resistance genes, organisms that express ESBL are frequently resistant to other antibiotic agents (Jacoby and Sutton, 1991). These plasmids are easily transmitted among bacteria and this account for ESBL producing isolates that are resistance to a variety of antibiotics. According to Podschu and Ullmann (Podschum and Ullmann, 1988), the multidrug resistant *Klebsiella* strain is unfortunately accompanied by a relatively high stability of the plasmids.

Spreading ESBL-producing strains is a concern, neonatal infants and babies less than 12 monthes are the major population easily infected by ESBL-producing Clinical isolates of *K. pneumoniae*. The rate of fatality is becoming higher in hospital, especially in intensive care units (Pessoa-Silva et al., 2003). Our study indirectly proved that PIF contaminated with *E. sakazakii* and *K. pneumoniae* may be the major culprit of infections among infants.

Conclusion

We recommend a focus on simple preventative strategies such as the promotion of breast milk feeding, inclusion of warnings on powdered infant formula packages that they may be contaminated with *E. Sakazakii*, and taking ultra-high-temperature (HUT) to kill pathogens such as *E. sakazakii*, *K. pneumoniae* and other Enterobacteriaceaes. In order to reduce the morbidity and mortality of infant infections caused by contaminated PIF, more technical specifications should be involved in PIF production in the future.

The Kirby-Bauer disk diffusion method is a very useful tools to evaluate the antibiotic susceptibility of bacteria. Double-disk synergy test (DDST) used for detecting ESBL is simple and effective to prove ESBL-producing in *E. sakazakii* and *K. pneumoniae*. In our study, 2 isolates (15.4%) from PIF were proved producing ESBL.

Collectively, present findings revealed that resistance to antibiotics in *E. sakazakii* and *K. pneumoniae* continued to spread from clinical infections to food like PIF. Multi-drug resistant and ESBL-producing strains

isolated from PIF are threatening infants all over the world, especially in the developing countries where antibiotics were extensively misused. How to prevent neonatal infections caused by *E. sakazakii*, *K. pneumoniae* and other Enterobacteriaceaes from PIF should be our main target in the future.

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