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Short communication: Multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex isolated from infant milk formula and utensils in a nursery in Rio de Janeiro, Brazil

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ABSTRACT

Infant milk formulas are not sterile products, and pathogenic bacteria can survive and multiply in these products. This study was performed, initially, to detect the presence of *Salmonella* spp. in reconstituted infant milk formula and on utensils previously sanitized used in their preparation or distribution in a nursery of a public hospital in Rio de Janeiro. None of the samples tested carried *Salmonella* spp. However, further identification of colonies growing on the selective media revealed the presence of several other gram-negative bacteria. Seventeen isolates were identified as belonging to *Acinetobacter baumannii-calcoaceticus* complex. Fourteen isolates presented a multidrug-resistance profile, by disc diffusion assays, and one of them—JE4—was also resistant to imipenem. The detection of *Acinetobacter* isolates in this work demonstrates inadequate hygiene practices in the preparation or distribution of infant milk formula.

Key words: *Acinetobacter* spp., multidrug resistance, infant milk formula, utensil

Short Communication

Concerns have been raised about the consumption of infant milk formulas (**IMF**) because the ingestion of contaminated formula or the use of improperly sanitized utensils in their preparation can be mechanisms of acquiring infections (Mammina et al., 2007). Multidrug-resistant gram-negative bacteria represent a major cause of infections in neonatal intensive-care units, and the feeding with infant formula is significantly associated with cross-transmission of resistant strains (Mammina et al., 2007; Mardaneh and Dallal, 2013). Many studies report the presence of different gram-negative bacteria in IMF, including *Acinetobacter* spp., but the attention given to this type of food was, in the most cases, focused only on *Cronobacter* spp. and *Salmonella* spp. (Wang et al., 2009; Miled et al., 2010; Abdullah Sani et al., 2013). *Salmonella enterica* is one of the most worrisome pathogens associated with IMF, because clear evidence exists that their presence can result in severe disease in at-risk population that consumes that food (Arsalan et al., 2013). The initial objective of this study was to detect the presence and the antibiotic resistance profile of *Salmonella* spp. and other gram-negative bacteria, if any, in reconstituted IMF samples and utensils previously sanitized used in their preparation or distribution from the nursery room of a public hospital in Rio de Janeiro, Brazil.

All the utensils—jars, spoons, baby bottles, trays, and rubber nipples—were sanitized by the nursery staff by washing with soap and water (with the aid of a brush, in the case of bottles) and subsequent immersion in boiling water for 10 min. No other sanitizing agent, such chlorine solution, was used. Reconstituted IMF from 3 different lots made for infants 0 to 6 mo of age were prepared in sterile heat-resistant containers with large volumes and subjected to heating in a conventional microwave oven for 45 s before distribution. Samples from utensils were obtained by swabs, and samples from IMF were collected in the original small containers. All of them were kept under refrigeration immediately conducted at the laboratory.

For samples of reconstituted IMF, preenrichment, selective enrichment, and plating were performed as described by Normative Instruction 62 (Brazil, 2003). Twenty-five milliliters of the IMF was homogenized with 225 mL of buffered peptone water (1%, wt/vol) and incubated for 24 h at 35°C. Selective enrichment was done by transferring 1 mL of this culture to Rappaport-Vassiliadis, tetrathionate, and selenite cystine broths (Himedia, São Paulo, Brazil) and incubating for 24 h at 41°C. Broths were then streaked on bismuth sulfite (Himedia), XLD (Himedia), and Rambach (Merck, Kenilworth, NJ) agar plates and incubated for 24 h at 35°C. Agar EMB (Himedia) was also included, aiming

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for the isolation of other gram-negative bacteria. Swabs collected were incubated in 9 mL of 1% (wt/vol) buffered peptone water at 37°C for 18 h before the selective enrichment and plating procedures at the same agar media described.

Atypical and typical colonies suggestive of *Salmonella* sp. were selected and submitted to conventional biochemical tests and slide agglutination with somatic polyvalent anti-*Salmonella* sera (Probac do Brasil, São Paulo, Brazil). The identification was confirmed by using the commercial panel Bactray (Laborclin, São Paulo, Brazil). *Salmonella enterica* ATCC 19214 was used as control.

Samples from spoons did not present colonies on the media used in this study. A total of 44 isolates were obtained from the other utensils and from the IMF analyzed. *Salmonella* sp. was not detected; however, different gram-negative species were identified. The presence of microorganisms was expected, because the cleaning of vessels was performed only by washing with soap and hot water immersion. Commercial sodium hypochlorite solution could also have been used. Reconstituted IMF provides an ideal environment for the growth of microorganisms. According to the FAO/WHO risk assessment (FAO/WHO, 2006), the number of microorganisms is dramatically reduced when IMF is reconstituted with water heated to at least 70°C. The evidence of contamination presented in this study suggests that, because large volumes are prepared and heated in microwave ovens, the internal temperature does not reach this minimum.

Acinetobacter baumannii-calcoaceticus (**ABC**) complex was the most isolated bacteria (17 isolates, 37.8%), followed by *Enterobacter cloacae* (12 isolates, 26.7%). Other species included *Hafnia alvei*, *Escherichia vulneris*, *Enterobacter aerogenes*, and *Proteus mirabilis*. The ABC complex isolates were found on the jars and also in 2 reconstituted IMF.

In this work, the differentiation of *A. baumannii* from the ABC complex was not performed. Even automated systems such as Phoenix (Becton Dickinson, Franklin Lakes, NJ) and Vitek2 and API 20NE (BioMérieux, Durham, NC) are not able to differentiate the species from the ABC complex (Abbott and Peleg, 2014). Useful tools involving the amplification by PCR of genes from carbapenem-hydrolyzing class D β-lactamases and the subunit B from DNA gyrase ($bla_{\text{OXA-51-like}}$ and *gyrB*) can help to identify members of this complex (Higgins et al., 2007; Hamouda, 2010; Gurung et al., 2013) and will be used in a future work.

The results were consistent with a study involving data from 7 countries about microorganisms isolated from IMF and utensils used in its preparation, in which *A. baumannii-calcoaceticus* and *E. cloacae* were the most found microorganisms (Chap et al., 2009).

The ABC complex organisms are found usually in water and soil. However, these microorganisms can cause infections such as pneumonia, urinary tract, surgical wound, and bloodstream infections (Blossom and Srinivasan, 2008; Barsoumian et al., 2013).

These pathogens are gaining importance because of the outbreaks reported and the infections caused in neonates. These patients are, in general, vulnerable, and the drug resistance presented by clinical isolates of this organism constitutes a real threat (Zarrilli et al., 2012).

Because of this fact, the 17 *Acinetobacter* isolates were submitted to antibiotic susceptibility testing by disc diffusion, performed according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2013). The following antibiotics (Sensifar, São Paulo, Brazil) were employed: amikacin (30 mg), ampicillin-sulbactam (10/10 mg), ceftazidime (30 mg), cefotaxime (30 mg), ciprofloxacin (5 mg), gentamicin (10 mg), imipenem (10 mg), tetracycline (30 mg), tobramycin (10 mg), and trimethoprim-sulfamethoxazole (1.25/23.75 mg). *Escherichia coli* ATCC 29522 was used as control. Results are presented in Table 1.

Resistance to ampicillin-sulbactam was observed in 15 (88.2%) isolates, whereas 14 (82.3%) isolates were resistant to cefotaxime and 12 (70.6%) to trimethoprim-sulfamethoxazole. The other tested antibiotics showed lower rates of resistance. Fourteen isolates were resistant to antibiotics belonging to at least 3 different classes, which confer to these bacteria a multidrug-resistant (**MDR**) profile (Magiorakos et al., 2012; Heizmann et al., 2013).

One of the MDR isolates—JE4—was also resistant to imipenem, an antibiotic from the carbapenems class. Imipenem and meropenem are considered the most effective antibiotics for the treatment of infections caused by this pathogen, and when the resistance to this class of antibiotics occurs, the treatment options become limited (Karageorgopoulos and Falagas, 2008; Joshi and Litake, 2013). Carbapenem-resistant *Acinetobacter baumannii* has emerged as a serious threat among ill neonates, because the bacteremia in these cases has a very high mortality rate (Thatrimontrichai et al., 2013).

The increasing of the antimicrobial resistance is providing therapeutic challenges, especially considering that most *A. baumannii* strains that are resistant to the carbapenems are also resistant to the many other antibiotics (Fishbain and Peleg, 2010). Carbapenem resistance may be conferred through various mechanisms, including the expression of lactamase carbapenemases, especially the group of the metallo-lactamases, able

Table 1. Source and antimicrobial-resistance profile of the isolates of *Acinetobacter baumannii-calcoaceticus* complex studied in this work

Source	Isolate	Resistance profile[1]	Growth on MDR *Acinetobacter* agar[2]
Jars	JE2	SAM, CTX, SXT	+
	JE3	AMI, SAM, CTX	+
	JE4	SAM, CTX, IMP, SXT	+
	JE5	AMI, SAM, CTX, GEN, SXT	+
	JE6	AMI, SAM, CIP, CTX, GEN, TET, SXT	+
	JE8	CIP, CTX, GEN, TOB, SXT	+
	JR1	SAM, CTX, GEN, SXT	+
	JR2	SAM, CIP, CTX, GEN, TOB	+
	JR3	SAM, CTX, TOB, SXT	+
	JR4	AMI, SAM, CTX, GEN, SXT	+
	JR5	SAM, CTX, SXT	+
	JR6	SAM, CTX, SXT	+
Baby bottles	MR1	AMI, SAM, CTX, SXT	+
	ME2	SAM, CTX, SXT	+
IMF[3] 1	AE1[4]	SAM	+
IMF 2	PR1[4]	—	−
	PR2	SAM, TET	−

[1]AMI = amikacin; CTX = cefotaxime; CIP = ciprofloxacin; GEN = gentamicin; IPM = imipenem; SAM = ampicillin-sulbactam; SXT = trimethoprim-sulfamethoxazole; TET = tetracycline; TOB = tobramycin.

[2]+, growth on selective agar; −, absence of growth or resistance to the antibiotics tested. All the experiments were repeated at least twice. MDR = multidrug resistant. The MDR *Acinetobacter* agar was from CHROMagar (Paris, France).

[3]IMF = infant milk formula.

[4]Non-MDR isolate.

to hydrolyze most of the lactams. The production of metallo-lactamases has been described in many *Enterobacteriaceae* strains and also in *Acinetobacter* spp. isolates (Maltezou, 2009; Lee et al., 2012). Metallo-lactamases producing ABC complex strains have become a global concern because these enzymes presented a high hydrolytic activity for the carbapenems, the most important group of antibiotics for the treatment of infections caused by MDR *Acinetobacter* (Bush and Jacoby, 2010; Roca et al., 2012).

For an additional confirmation, and to seek a practical and rapid method for the detection of MDR *Acinetobacter* strains in the nursery, a selective chromogenic medium (MDR *Acinetobacter*, CHROMagar, Paris, France) was used to identify multidrug-resistant isolates by the growth in prominently red colonies, after overnight incubation. All of the 14 multidrug-resistant isolates detected in the disc diffusion assay were also identified as MDR by growth in this medium (Table 1). However, one isolate, AE1, classified as non-MDR, was also able to grow, suggesting that this isolate is probably resistant to other antibiotics not tested in this work. Barsoumian and coworkers (2013) also related that this chromogenic agar permits the rapid detection of MDR *Acinetobacter* spp., although is unable to distinguish carbapenem-resistant from carbapenem-susceptible strains.

The detection of MDR *Acinetobacter* isolates in this work demonstrates that inadequate hygiene of the utensils could represent a significant risk of the transmission of this multidrug-resistant pathogen to neonates related to the consumption of IMF, reflecting poor infection-control procedures. To solve this problem, educational training emphasizing good manufacturing practices and food safety should be introduced to the staff of the nurseries, aimed at the identification and correction of the critical control points.

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