ENTEROBACTER CLOACAE ISOLATED FROM UTENSILS USED IN BABY FEEDING IN A MILK DISPENSARY IN RIO DE JANEIRO

W. G. R. Nascimento, M. S. Moraes, B. C. Araújo, B. V. Souza, J. O. R. Farias; D. H. Melo, J. S. Nascimento*

Laboratory of Microbiology, Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, Rua Senador Furtado 121/ Lab 412, 20270-021, Rio de Janeiro, Brazil. Phone: (21) 2566-7792 - *e-mail: janaina.nascimento@ifrj.edu.br

RESUMO – Diferentes bactérias da família Enterobacteriaceae têm sido frequentemente isoladas de fórmulas lácteas infantis (FLI), incluindo Enterobacter cloacae, que é capaz de colonizar o sistema gastrintestinal humano e é, portanto, visto como um importante patógeno. Neste trabalho, doze isolados de bandejas de plástico e bicos de mamadeiras reutilizáveis utilizados na alimentação de bebês em um lactário foram identificados como E. cloacae. Seis isolados exibiram um perfil de multirresistência e nove foram capazes de produzir biofilme, comparable com o controle positivo. Todos os isolados expressaram os fenótipos de produção de β-lactamase de espectro estendido e de sensibilidade reduzida aos carbapenens pelo método testado. Os 12 isolados também foram sensíveis às concentrações de hipoclorito de sódio testadas após exposição por 30 segundos, no entanto, na presença de matéria orgânica, a susceptibilidade diminuiu significativamente. Os resultados indicam a necessidade de maior atenção para o preparo e distribuição de FLI e especialmente para a higienização de utensílios, que podem se tornar veículos de transmissão de agentes patogênicos resistentes, como E. cloacae.

ABSTRACT – Different bacteria of the Enterobacteriaceae family have been frequently isolated from infant milk formula (IMF), including Enterobacter cloacae, which is capable of colonizing the human gastrointestinal tract, are therefore viewed as an important pathogen. In this work, twelve isolates from plastic trays and reusable bottle nipples used for feeding babies in a milk dispensary were identified as E. cloacae. Six isolates exhibited a typical multidrug resistant profile and nine were able to produce biofilm, comparable to the positive control. All isolates expressed extended spectrum β-lactamase and reduced susceptibility to carbapenens phenotypes by the used methods. The 12 isolates were also susceptible to the sodium hypochlorite concentrations tested after exposure for 30 seconds, but in presence of organic matter, the susceptibility decreased significantly. The results indicate the need for attention to the preparation and distribution of IMF and especially during the sanitation of utensils, which can become vehicles of transmission of resistant pathogens such as E. cloacae.

PALAVRAS-CHAVE: Enterobacter cloacae; multirresistência a drogas; utensílios; hipoclorito de sódio

KEYWORDS: Enterobacter cloacae; multidrug resistance; utensils; sodium hypochlorite.
1. INTRODUCTION

According to the World Health Organization, the contamination of infant milk formulas (IMF) can occur intrinsically, or from extrinsic sources. The intrinsic contamination occurs during its manufacture, and the extrinsic contamination may occur when contaminated utensils are used during preparation, storage or distribution of formulas (WHO, 2007).

The microbiological safety of IMF is an item of great importance, since newborns lack an immune system developed and a competitive intestinal microbiota, especially those who depend on the services of a milk dispensary. Pathogenic bacteria from Enterobacteriaceae family have been frequently isolated from IMF. Cronobacter sp. (formerly Enterobacter sakazakii) is the most important pathogen (Oonaka et al., 2010; Yao et al., 2012; Arsalan et al., 2013), however, *E. cloacae* is able to colonize the human gastrointestinal tract and is considered an important pathogen, particularly in nurseries and neonatal critical care, especially in regard to drug resistance (Oteo et al., 2013; Stoesser et al., 2015).

In a previous work performed by our group, a total of forty-four isolates were obtained from utensils (jars, spoons, baby bottles, trays and rubber nipples – all reusable) and IMF from a nursery in Rio de Janeiro, Brazil (Araújo et al., 2015). Twelve isolates (from baby bottles and rubber nipples) were identified as *Enterobacter cloacae* and had not been investigated so far. In the present work, these isolates were tested for antibiotic resistance, qualitative production of biofilm and susceptibility to sodium hypochlorite, aiming to evaluate the pathogenic potential of these isolates.

2. MATERIAL AND METHODS

2.1 Isolates

The twelve isolates were obtained from utensils of a milk dispensary in Rio de Janeiro city and identified as described in a previous study (Araújo et al., 2015). Samples from jars, spoons, baby bottles, trays and rubber nipples – all reusable) and IMF from a nursery in Rio de Janeiro, Brazil (Araújo et al., 2015). Twelve isolates (from baby bottles and rubber nipples) were identified as *Enterobacter cloacae* and had not been investigated so far. In the present work, these isolates were tested for antibiotic resistance, qualitative production of biofilm and susceptibility to sodium hypochlorite, aiming to evaluate the pathogenic potential of these isolates.

2.2 Antibiotic resistance

The antibiogram was performed according to CLSI (2015), using the following antibiotics (Sensifar, Sao Paulo, Brazil): amikacin (30 μg), ampicillin-sulbactam (10/10 μg), ceftazidime (30 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), tetracycline (30 μg), tobramycin (10 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg). The production of extended spectrum β-lactamase (ESBL) and the reduced susceptibility to carbapenems (KPC) were evaluated by growth on Chromagar ESBL™ and KPC™ agar.

2.3 Qualitative evaluation of biofilm production

Biofilm formation was evaluated by the Congo red method, based on the enhancement of the exopolysaccharide production, performed according Freeman et al.(1989), using the biofilm producer strain *Salmonella enterica* ATCC 14028 as positive control.

2.4 Resistance to chlorine
Chlorine solutions were prepared using a sodium hypochlorite containing 2% of active chlorine solution diluted in distilled water (v/v), and concentration was adjusted to 200, 100 and 50 ppm. The isolates were grown in TSA plates and colonies were inoculated in saline (0.85% NaCl [w/v]) until the concentration of approximately \(1.5 \times 10^8\) UFC/ml. Aliquots were added to the active chlorine solution until the final concentration of \(1.5 \times 10^6\) UFC/ml and the viability of cells was evaluated over time under room temperature. To simulate the presence of organic matter, the same chlorine solutions were prepared in Casoy broth.

3. RESULTS AND DISCUSSION

Disregarding the intrinsic resistance of \(E.\) cloacae to ampicillin and amoxicillin-clavulanic acid combination, 6 isolates (BA7, BIE1, BIE2, BIE3, BIE7 and BIR4) were resistant to antibiotics belonging to at least 3 different classes, conferring to these isolates a multidrug-resistant (MDR) profile (Magiorakos et al., 2012; Heizmann et al., 2013). BIR4 presented the broader antibiotic resistance profile. Results are presented on Table 1.

### Table 1: Characteristics of the \(Enterobacter\) cloacae isolates studied in this work

<table>
<thead>
<tr>
<th>Utensil sample</th>
<th>Isolate</th>
<th>Antibiotic resistance profile</th>
<th>Biofilm production(^b)</th>
<th>Phenotype KPC(^c)</th>
<th>Phenotype ESBL(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trays</td>
<td>BA1</td>
<td>Ame*, Cfl</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BA3</td>
<td>Amp*, Ame*, Cip</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BA5</td>
<td>Amp*, Ame*, Cfl, Ipm</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BA6</td>
<td>Ame*, Ctx, Cfl</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BA7</td>
<td>Ame*, Ctx, Cfl, Ipm, Tri</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Bottle nipples</td>
<td>BIE1</td>
<td>Ame*, Cfl, Cip, Tet</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BIE2</td>
<td>Ame*, Cfl, Tet, Tri</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BIE3</td>
<td>Ame*, Cfl, Ctx, Tet, Tri</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BIE5</td>
<td>Ame*, Cfl, Tri</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BIE7</td>
<td>Ame*, Cfl, Cip, Tet</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BIE8</td>
<td>Ame*, Cfl, Cip</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>BIR4</td>
<td>Ame*, Amp*, Atm, Cfl, Ctx, Clo, Tob, Tri</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
</tbody>
</table>

AMC, amoxicilin-clavulanic acid; AMP, ampicillin, ATM, aztreonam; CFL, ceflofloxin; CTX, cefotaxime; CLO, cloranfenicol; IPM, imipenem; TRI, trimethoprim. None of the isolates was resistant to amikacin, ceftazidime, ciprofloxacin, gentamicin, streptomycin, norfloxacin, tetracycline or tobramycin; *, intrinsic resistance.

Since resistance to cephalosporins (cephalotin and cephotaxime), monobactan (aztreonam) and also to a carbapenem (imipenem), was observed, the isolates were also tested for expression of reduced susceptibility to carbapenems and for production of extended-spectrum beta-lactamases by the growth on Chromagar KPC™ and Chromagar ESBL™, respectively. All the isolates presented positive results to the both phenotypes (metallic blue colonies).

The biofilm formation on equipment surfaces used in infant formula preparation, processing plants or even in feeding areas may increase the risk of infections to infants. Although there are other methods for detecting biofilm, such as tube adherence and microtitre plate methods, both involving staining with crystal violet, this method was choose because is rapid, less expensive, and reproducible, besides have the advantage of the colonies remaining viable on the medium (Niveditha et al., 2012; Hedayati et al., 2014).

Nine of the isolates were able to produce biofilm (Table 1) comparable to the positive control, adding more items to the pathogenic potential of these bacteria. In general, the biofilm formation by \(E.\) cloacae have been most studied on clinical isolates as a result of their ability to colonize medical devices (Donlan et al., 2001; Revdiwala et al., 2012; Nyenje et al., 2013) but these results indicates that the capacity of this pathogen to adhere to feeding bottles and other food utensils deserves special
attention, principally because when bacterial cells are enmeshed in biofilms, their removal or inactivation on inert surfaces by washing with water or treatment with sanitizers is not always achieved (Kim et al., 2006; Moraes et al., 2015).

Our research group has recovered bacteria from IMF and utensils used in its preparation from nurseries in Rio de Janeiro, Brazil, and have observed that inadequately sanitized food-contact surfaces as baby bottles, bottle nipples, trays and spoons have been implicated as sources of contamination (Moraes et al., 2015). Sodium hypochlorite is the most widely used commercial sanitizer and the susceptibility of the *E. cloacae* isolates to this agent was investigated. All the 12 isolates were susceptible to the chlorine solutions after exposition for up to 30 seconds.

Reusable plastic, however, with time of use, end up deteriorating and and may have small grooves and cracks that, if not properly sanitized, can accumulate organic matter and facilitate microbial growth. Therefore, to simulate the presence of organic matter, the same chlorine solution was prepared in Casoy broth. The presence of a source of organic matter resulted in a dramatic reduction in the sensitivity of the isolates tested. All of them presented growth similar to the positive control, even after 24 hours of incubation at room temperature. Two isolates, BIE1 and BIR4, were susceptible to the minimum concentration of 2,500 ppm of active chorine, while the other 11 isolates were inhibited by only 5,000 ppm. These results suggest that the presence of organic matter significantly interfere in the antimicrobial action of sodium hypochlorite. Similar results by Adikesavalu and collaborators (2015), using tryptic soy broth as diluents for different sanitizers to verify inhibition strains *Edwardsiella tarda*.

4. CONCLUSIONS

The presence of MDR *E. cloacae* in the utensils as plastic trays and rubber nipples demonstrate a critical point in the process of sanitization of baby feeding utensils and these failures can endanger the health of infants. The results emphasize the need to use disposable bottle nipples (and also other utensils, like baby bottle and trays), since reusable can present cracks capable of accumulating organic matter and bacteria that are difficult to remove, even in the presence of sanitizers. In establishments where the use of disposable utensils is not possible, the replacement of plastic bottles and trays for glass or metal could contribute to the reduction of biofilm formation by facilitating the sanitization and thereby prevent the persistence of pathogens such as *E. cloacae*.

5. REFERENCES


