Clonality and Antimicrobial Resistance Gene Profiles of Multidrug-Resistant Salmonella enterica Serovar Infantis Isolates from Four Public Hospitals in Rio de Janeiro, Brazil

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In Brazil, Salmonella enterica serovar Infantis resistant to various antimicrobials, including cephalosporins, has been identified as an etiological agent of severe gastroenteritis in hospitalized children since 1994. In this study, 35 serovar Infantis strains, isolated from children admitted to four different Rio de Janeiro, Brazil, hospitals between 1996 and 2001, were characterized by pulsed-field gel electrophoresis (PFGE) and antimicrobial susceptibility testing in order to determine their genetic relatedness and antimicrobial resistance profiles. Thirty-four serovar Infantis strains were resistant to at least two antibiotic classes, and all 35 strains were susceptible to fluoroquinolones, cephemycins, and carbapenem. Extended-spectrum beta-lactamase (ESBL) screening by double-disk diffusion indicated that 32 serovar Infantis strains (91.4%) produced beta-lactamases that were inhibited by clavulanic acid. Antimicrobial resistance gene profiles were determined by PCR for a subset of 11 multidrug-resistant serovar Infantis strains, and putative ESBLs were detected by isoelectric focusing. Ten serovar Infantis strains carried blaTEM, catI, ant(3')Ia and/or ant(3')Ib, sulI and/or sulIII, and tet(D) genes as well as an integron-associated aac(6')-Iq cassette. Eight strains possessed at least four different beta-lactamases with pl profiles that confirmed the presence of both ESBLs and non-ESBLs. Our PFGE profiles indicated that 33 serovar Infantis strains isolated from Rio de Janeiro hospitals came from the same genetic lineage.

For many years, ampicillin, sulfamethoxazole-trimethoprim, and chloramphenicol were the drugs of choice for the treatment of severe Salmonella infections, but increasing rates of resistance to these agents have significantly reduced their efficacies (28, 35). Subsequently, third-generation cephalosporins, due to their pharmacodynamic properties as well as low resistance levels in Salmonella, are being used to treat invasive salmonellosis (5, 11).

In 1994, Asensi and Hofer reported the presence in Rio de Janeiro, Brazil, of Salmonella enterica serovar Infantis strains that were resistant to a growing number of antimicrobial agents (6). Two years later, a nosocomial outbreak in a neonatal unit of one hospital (designated HC) was reported by De Moraes et al. (13). The authors detected multidrug-resistant serovar Infantis phenotypes, including resistance to broad-spectrum cephalosporins that was transferred by a plasmid of 148 kbp. An investigation carried out from 1998 to 1999 reported an infection due to extended-spectrum beta-lactamase (ESBL)-producing serovar Infantis in the neonatal unit of a public hospital (HC) in Rio de Janeiro, Brazil, indicating inadequate infection control practices and nursery overcrowding (30). Since then, multidrug-resistant serovar Infantis has been isolated in three other public health hospitals (designated HA, HB, and HD) of Rio de Janeiro, Brazil. Two are pediatric reference hospitals that often see children from the western and northern regions of the city, where parts of the population have lower socioeconomic and sanitary conditions. Some children were human immunodeficiency virus positive, and most suffered from recurring infections and had histories of rehospitalization. Although HC is a university-affiliated hospital and HD is a reference hospital for cancer, both provide medical care for patients with debilitating diseases such as AIDS and diabetes. In addition, these patients are subjected to prolonged hospitalizations that are often accompanied by the empirical use and sometimes overuse of antimicrobial drugs (ampicillin and/or cephalosporins and/or aminoglycosides). This led us to monitor the prevalence and antimicrobial susceptibility of serovar Infantis in hospitals in Rio de Janeiro, Brazil. The aims of this research were to (i) determine the antimicrobial susceptibility patterns, (ii) identify the main mechanisms involved in antimicrobial resistance, (iii) ascertain the presence and spread of integron-carried resistance genes, and finally, (iv) assess the macro-restriction fragment length polymorphisms between multidrug-resistant serovar Infantis strains from those hospitals.

MATERIALS AND METHODS

Bacterial strains. Serovar Infantis strains were isolated according to the method of Costa and Hofer (12), and the antigenic characterization was based on the Kauffmann-White scheme described by Poppof (29a). This study included 35
serovar Infantis strains isolated from the stools or blood of children under 7 months who were admitted to four public hospitals (HA, HB, HC, and HD) in Rio de Janeiro, Brazil, from 1996 to 2001. Only one isolate per patient was included in the study.

**Antimicrobial susceptibility testing and extended-spectrum beta-lactamase assay.** Disk diffusion tests were performed according to Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) recommendations by using disks (Oxoid Limited, Hampshire, England) impregnated with ampicillin (AMP; 10 μg), aztreonam (ATM; 30 μg), cephalothin (CEF; 30 μg), ceftazidime (CAZ; 30 μg), cefotaxime (CTX; 30 μg), cefotaxime (CRO; 30 μg), cefazidime (CAZ; 30 μg), cefoxitin (FOX; 30 μg), cefuroxime (CXM; 30 μg), ceftazidime (CAZ; 30 μg), trimethoprim-sulfamethoxazole (SXT; 25 μg), and tetracycline (TET; 30 μg).

**Preparation of crude protein extracts and IEF.** The 11 ESBL-positive isolates were grown in 2 ml of Mueller-Hinton broth at 37°C overnight, and cells were harvested by centrifugation at 16,000 × g for 2 min. After discarding the supernatant, cells were resuspended in 250 μl of 1% glycine and 30% glycerol and were sonicated twice for 30 s, with cooling of the cells on ice between sonications. Cell lysates were centrifuged at 16,000 × g for 15 min. Supernatants were collected into clean tubes and stored at −20°C. Prior to isoelectric focusing (IEF), cell extracts were tested for beta-lactamase activity by adding 50 μl of 0.1% nitrocefin stock solution (Oxoid Limited, England) to 17 μl of extract and then recording the time required for the reaction to turn dark pink. The optimal reaction time was 30 to 120 s. For reaction times of 5 s or less, the extract was diluted with phosphate buffer and retested. For isolates with reaction times of 5 min or more, another extract was prepared from a culture of greater density and was sonicated twice for 30 s, with cooling of the cells on ice between sonications. Cell lysates were centrifuged at 16,000 × g for 15 min. Supernatants were collected into clean tubes and stored at −20°C.

**Integron 5’CS/3’CS**

<table>
<thead>
<tr>
<th>Gene (or integron)</th>
<th>Primer sequence 5’ to 3’</th>
<th>Reference strain( plasmid)</th>
<th>Source (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetA</td>
<td>F, GCT ACA TCC TGC TGG CTC TT; R, CAT AGA TCG CCG TGA AGA GG</td>
<td>E. coli D20-15 (pSL18)</td>
<td>S. Levy (21)</td>
</tr>
<tr>
<td>tetB</td>
<td>F, TTT GGG AGG GGC AAG TTT TG; R, GTA ATG GCC CAA TAA CAC AG</td>
<td>E. coli D20-16 (pRT11)</td>
<td>S. Levy (20)</td>
</tr>
<tr>
<td>tetC</td>
<td>F, CTT GAG AGC CTT CAA CCC AG; R, ATG GTC GTG ATC ATC TAC CGT CC</td>
<td>E. coli D20-6 (pBR322)</td>
<td>S. Levy (20)</td>
</tr>
<tr>
<td>tetD</td>
<td>F, AAA CCA TTA CCG CAT TCT GC; R, GAC CGG ATA CAC CAT CCA TC</td>
<td>E. coli D22-2 (pSL106)</td>
<td>S. Levy (20)</td>
</tr>
<tr>
<td>tetE</td>
<td>F, AAA CCA CAT CTT CCA TAC GC; R, AAA TAG GCC ACA ACC GTC CC</td>
<td>E. coli D22-14 (pSL1504)</td>
<td>S. Levy (19)</td>
</tr>
<tr>
<td>tetG</td>
<td>F, CAG CTT TCG GAT TGT TAC GG; R, GAT TGG TGA GCC TGG TTA GC</td>
<td>E. coli HB101 (pJ8122)</td>
<td>T. Aoki (37)</td>
</tr>
<tr>
<td>tetH</td>
<td>F, CCT GAA AAC CAA ACT GCC TC; R, ACA GAC CAT CCC AAT AAG CG</td>
<td>Pasteurella multocida (pVM112)</td>
<td>M. Roberts (15)</td>
</tr>
<tr>
<td>catI</td>
<td>F, TCA GCT GGA TAT TAC GGC CT; R, CAT TCT GGC GAC ATG AGA G</td>
<td>LK 169 (pBR329)</td>
<td>2</td>
</tr>
<tr>
<td>catII</td>
<td>F, ATT CAG CCT GAC CAC CAA AC; R, CTT CCT GCT GAA ATG AGA G</td>
<td>E. coli J52 (pSA)</td>
<td>M. Roberts (25)</td>
</tr>
<tr>
<td>catIII</td>
<td>F, CCC ACA ATT CAT CGT ACC CT; R, GAA CTT GTA CTG AGA GCG GC</td>
<td>E. coli J53 (R387)</td>
<td>M. Roberts (24)</td>
</tr>
<tr>
<td>sulI</td>
<td>F, SAC CGC GGC GAT CGA AAT GC; R, GGT TTC CGA GAA GGT G</td>
<td>820 Proteus mirabilis</td>
<td>P. H. Roy (18)</td>
</tr>
<tr>
<td>sulII</td>
<td>F, ATC GCT CAT TTT CCG CA; R, CTC GTG CTT GCG CAT GAG AG</td>
<td>Serovar Typhimurium CO-8861</td>
<td>C. Clark (31)</td>
</tr>
<tr>
<td>DhfrI</td>
<td>F, CGA AGA ATG GAG TTA TCG GC; R, TAA ACA TCA CCT TGC GGC TC</td>
<td>C600 (R483)</td>
<td>32</td>
</tr>
<tr>
<td>aadA1</td>
<td>F, GCG CTA AAT GAA ACC TTA AC; R, TCG CCT TTC AGC TAG TGC AG</td>
<td>E. coli JE 2571 (pHH1457)</td>
<td>D. Taylor (9)</td>
</tr>
<tr>
<td>aadA2</td>
<td>F, TGT TGG TTA CTG TGG CCA TA; R, GCT GCG AGT TCC GTA ATC GCC TC</td>
<td>Serovar Typhimurium PT104 96-5227</td>
<td>D. Taylor (7)</td>
</tr>
<tr>
<td>aph5’Ia</td>
<td>F, TTA TGC TTC TTC CGA CCA TC; R, GAG AAA ACT CAC CGA GCC AG</td>
<td>E. coli JE 2571 (pHH1457)</td>
<td>D. Taylor (9)</td>
</tr>
<tr>
<td>aac6’Iq</td>
<td>F, GCT GGA AAT GAA TCA TGG GT; R, TAC TTT CCC TAC CCT TGC CT</td>
<td>BR-SA-97-368</td>
<td>D. Rodrigues (23)</td>
</tr>
<tr>
<td>b1aTEM-1</td>
<td>F, ATA AAA TTA TTT TGG AAC AAA; R, AGC AGT TAC CAA TGC TTA ATC A</td>
<td>Neisseria gonorrhoeae 18795</td>
<td>14</td>
</tr>
<tr>
<td>Integron 5’CS/3’CS</td>
<td>F, GGC ATC CAA GCA GCA AG; R, AAG CAG ACT TGA CCT GA</td>
<td>Serovar Typhimurium PT104 96-5227</td>
<td>D. Taylor (18)</td>
</tr>
</tbody>
</table>

*a* F, forward primer; R, reverse primer.

*b* The reference strain served as a positive control for PCRs.
PFGE: macro restriction fragment patterns of Salmonella serovar Infantis genome digested with SpeI. Lanes: M, molecular weight marker of Salmonella Branderup strains; A2, PFGE profile of 3 HD strains; C, PFGE profile of a midwestern hospital strain; B, PFGE profile of a hospital strain; A1, PFGE profile of 18 HA, 4 HB, 5 HC strains; A3, PFGE profile of 1 HA strain; A5, PFGE profile of 1 HA strain; A4, PFGE profile of 1 HA strain.

FIG. 1. PFGE: macro restriction fragment patterns of Salmonella serovar Infantis genome digested with SpeI. Lanes: M, molecular weight marker of Salmonella Branderup strains; A2, PFGE profile of 3 HD strains; C, PFGE profile of a midwestern hospital strain; B, PFGE profile of a northern hospital strain; A1, PFGE profile of 18 HA, 4 HB, 5 HC strains; A3, PFGE profile of 1 HA strain; A5, PFGE profile of 1 HA strain; A4, PFGE profile of 1 HA strain.

FIG. 2. PFGE macro-restriction fragment polymorphism.

results and discussion

Many researchers are successfully using PFGE to investigate the epidemiologies of strains involved in outbreaks caused by beta-lactamase- and ESBL-producing bacteria (8). The PFGE analysis of the 35 serovar Infantis strains resulted in five PFGE restriction fragment profiles (Fig. 1 and 2). The comparative evaluation of the PFGE profiles yielded four fragment patterns (A1, A3, A4, and A5) for HA isolates. Three HB and five HC
isolates had the PFGE profile A1 that was also encountered in 18 HA strains. The three HD strains (PFGE profile A2), isolated in 2001, showed 95% similarity to PFGE profiles A1 and A3 (Fig. 1). Macro-restriction fragment patterns of strains from northern (PFGE profile B) and midwestern (PFGE profile C) regions of Brazil were completely different from those of strains from Rio de Janeiro, Brazil.

The A1 profile, found in 26 strains isolated from 1996 to 2001 in HA, HB, and HC, was considered to be the PFGE profile associated with the MDR serovar Infantis outbreaks. The PFGE patterns of serovar Infantis strains were then classified according to their similarities to the outbreak pattern. Those patterns that differed by at least two fragments were classified as unrelated types by considering that they derived from two genetic events and their isolating origins.

The susceptibility profiles of serovar Infantis are shown in Table 2. All of the strains were susceptible to carbapenem (imipenem), ciprofloxacin, nalidixic acid, and cephaparin (cefoxitin). All of the strains, except for one, were resistant to ampicillin, and most were resistant to cephalosporins (including extended spectrum). It is interesting that strains resistant to the highest number of antimicrobials (resistance profile ACSSuTTmKG, etc. [Table 2]) had similar PFGE profiles and were isolated from 1996 to 2001 from patients in different hospitals. The high prevalence of resistance to these particular antimicrobials may be due to selective pressure since these antimicrobials, with the exception of kanamycin and streptomycin, are among the agents most often prescribed in these hospitals. Resistance to kanamycin and streptomycin, however, may have been acquired through horizontal gene transfer since aminoglycoside resistance genes are often found on plasmids and transposons that encode resistance determinants for other classes of antimicrobials (34, 36). Tetracycline resistance (97.2%) and aztreonam resistance (96.1%) were also common among the multidrug-resistant strains. It is not surprising that the four hospitals involved in this study experienced great difficulties in deciding which antimicrobials to use for treatment. The implementation of effective screening methods for the detection of beta-lactamases and ESBLs as well as the establishment of surveillance programs became key factors in the control of hospital outbreaks (16).

PCR detection of resistance genes in nine isolates resistant to five classes of antimicrobials, represented by ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline, showed that all of the strains with the ACSSuTTmKG resistance profile carried blaTEM, catI, aadA1, sulI, sulII, and tet(D) resistance genes and an integron containing an aac(6’)-Ia gene cassette that codes for amikacin resistance (Table 3). The only variation among these strains was the
TABLE 3. Antimicrobial resistance genes detected in multidrug-resistant serovar Infantis strains

<table>
<thead>
<tr>
<th>Resistance profilea</th>
<th>No. of strains</th>
<th>Integron geneb</th>
<th>PFGE profile</th>
<th>Antimicrobial resistance genesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACSSuTTmKG (ATM, CEF, CXM, CAZ, CTX, CRO, FEP)</td>
<td>6</td>
<td>aac(6')-Iq</td>
<td>A1</td>
<td>blaTEM, catI, aadA1, sulII, tet(D)</td>
</tr>
<tr>
<td>ACSSuTTmKG (ATM, CEF, CXM, CAZ, CTX, CRO, FEP)</td>
<td>1</td>
<td>aac(6')-Iq</td>
<td>A1</td>
<td>blaTEM, catI, aadA1/A2, sulII, tet(D)</td>
</tr>
<tr>
<td>ACSSuTTmKG (ATM, CEF, CXM, CTX, CAZ, FEP)</td>
<td>1</td>
<td>CAZ</td>
<td>A1</td>
<td>blaTEM, catI, aadA1, sulII, tet(D)</td>
</tr>
<tr>
<td>ACSSuTTmKG (ATM, CEF, CXM, CTX, CRO, FEP)</td>
<td>1</td>
<td>aac(6')-Iq</td>
<td>A1</td>
<td>blaTEM, catI, aadA1/A2, sulII, tet(D)</td>
</tr>
<tr>
<td>ASSuTKG (ATM, CEF, CXM, CAZ, CTX, CRO, FEP)</td>
<td>1</td>
<td>aac(6')-Iq</td>
<td>A5</td>
<td>blaTEM, catI, aadA1/A2, sulII, tet(D)</td>
</tr>
</tbody>
</table>

a Cephalosporin and aztreonam resistance profiles are shown in parentheses. A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfamethoxazole; T, tetracycline; Tm, trimethoprim; K, kanamycin; G, gentamicin.

b The integron cassette size was 1,269 bp.

c "aadA1/A2" or "sulI/II" indicates the presence of both aadA1 and aadA2 or both sulI and sulII, homologous genes, respectively, within a strain.

d This strain was immediately resistant to streptomycin.

presence or absence of the streptomycin/spectinomycin resistance gene aadA2, also known as ant(3)'lb, a variant of the gene aadA1 [ant(3)lA]. The serovar Infantis strain with the ACSSuTTmKG resistance profile was immediately resistant to streptomycin and yet carried both aadA1 and aadA2. Redundancy of resistance genes was also detected in 10 strains carrying two sulfonamide resistance genes, sulI and sulII.

Thirty-two (91.4%) serovar Infantis strains were classified as clavulanic-acid-inhibited ESBL-producing strains according to CLSI standards (21 from HA, 3 from HB, 5 from HC, and 3 from HD). Twenty-two strains (62.8%) were resistant to both CTX and CAZ (Table 2), which suggested the presence of at least one ESBL. According to the beta-lactamase classification scheme of Bush et al. (1995), cefotaximases are class A ESBLs (group 2b) that generally have higher hydrolytic activities against cefotaxime than ceftazidime, while ceftazidimases (also group 2be ESBLs) generally hydrolyze ceftazidime more readily than cefotaxime (10). In addition, group 2be ESBLs inactivate not only extended-spectrum cephalosporins but also monobactams such as aztreonam. Ten of the serovar Infantis strains characterized in this study were resistant to both CTX and CAZ, while only one was resistant to only CTX (Table 3). DNA sequencing of the amplicons obtained with blaTEM primers (which targeted the conserved region of TEM-related enzymes) revealed the presence of the non-ESBL blaTEM-1. In order to determine whether more than one beta-lactamase was produced by these 11 multidrug-resistant serovar Infantis strains, isoelectric focusing was performed (Table 4).

The pI profiles indicated the presence of beta-lactamases with pI values of 5.4, 6.3, 6.9, and 9.0. The six strains with the antibiogram ACSSuTTmKG (resistance profile, ATM, CEP, CXM, CAZ, CTX, CRO, FEP) had at least four different beta-lactamases (since there could be more than one beta-lactamase present in a strain with the same pI value), while another strain with the same resistance profile produced only two types of beta-lactamases (pI values 9.0 and 5.4). This result is significant since all seven strains are resistant to CEP, CXM, CAZ, CTX, CRO, and FEP, indicating that resistance to those cephalosporins requires the presence of only two types of beta-lactamases with pI values of 5.4 and 9.0. In addition, those seven strains are also resistant to the monobactam ATM, which indicates, according to Bush et al., that a group 2be ESBL is present within the strain (10).

The presence of identical antimicrobial resistance genes and the close relatedness of strains as determined by PFGE analysis provides evidence that the hospitals involved in this study had a salmonellosis outbreak that was caused by serovar Infantis strains that shared the same phylogenetic lineage. It is important to emphasize that strains from HC were isolated in only 1996, while strains from HB were isolated in 1997 and 1999. HA strains were isolated from 1996 to 1999. At the beginning of 2001, HD was informed about the characteristics and clonal nature of multidrug-resistant serovar Infantis so that appropriate control measures could be developed and, subsequently, serovar Infantis was no longer detected in the hospital environment. The guidelines and rules that provide for the planning of the National Program of Hospital Infection Control were defined by administrative rule GM 2.616 as of 12 May, 1998. This decree categorizes children hospitalized in high-risk nurseries as intensive-care patients requiring particular attention to infections due to multidrug-resistant pathogens (22). These patients are subjected to standard procedures for controlling nosocomial infections, such as the cleaning and disinfection of medical equipment, frequent hand washing, patient-to-patient contact precautions, and the monitoring of patients’ stools for the presence of multidrug-resistant serovar Infantis. The best strategy for antimicrobial therapy and specific infection control measures for each patient was determined on a case-by-case basis (1).

The results in this study indicate that efficient surveillance programs and effective decontamination procedures must be...
implemented for the prevention of nosocomial outbreaks of salmonellosis caused by multidrug-resistant serovar Infantis.

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