DISSEMINATION OF SALMONELLA ENTERICA SEROTYPE AGONA AND MULTIDRUG-RESISTANT SALMONELLA ENTERICA SEROTYPE TYPHIMURIUM IN CUBA

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Abstract. The molecular epidemiology, antimicrobial susceptibility, and mechanisms of resistance of 34 Salmonella spp. strains causing acute gastroenteritis, isolated from different regions in Cuba, were determined. Sixty-four percent of the strains showed multiresistance. Salmonella typhimurium was the most frequent with 15 strains (44%), 13 of which belonged to phagotype 104 and presented similar genetic profiles of pulsed field gel electrophoresis. High levels of resistance to tetracycline (53%), spectinomycin (50%), ampicillin (44%), and chloramphenicol (41%) were found. Resistance to tetracycline was associated with the tet G and tet A genes. Resistance to ampicillin was caused by the presence of β-lactamases, mainly the CARB type. The floR gene was the main mechanism of resistance to chloramphenicol. Our results showed an antimicrobial susceptible clone of Salmonella enterica serotype Agona in two separate regions. This is the first report of the widespread dissemination of a multiresistant clone of S. enterica serotype Typhimurium definitive phage type 104 in Cuba.

INTRODUCTION

In both developing and developed countries, acute gastroenteritis causes high levels of morbidity and mortality. One of the main etiological agents of this disease is Salmonella spp. The Salmonella genus is composed of approximately 2,400 serotypes with different phenotypic and genotypic characteristics. One of these characteristics is antimicrobial resistance. The Salmonella genus has been traditionally susceptible to antimicrobial agents, but an increase in the levels of resistance worldwide has been observed. Salmonella infections in Cuba is the third most frequent causes of acute gastroenteritis in adults and children after rotavirus and Entamoeba histolytica.

In Cuba, several studies have reported a high percentage of Salmonella spp. strains resistant to several antimicrobial agents such as ampicillin, chloramphenicol, nalidixic acid, and trimethoprim/sulphamethoxazole.

An efficient route of acquisition of resistance is through genetic elements including plasmids, transposons, and integrons. Currently, nine classes of integrons have been described, with the class 1 integron being the most frequently detected in clinical strains. Integrons have been described as an important cause of the spread of antimicrobial resistance in a variety of enteric bacteria including Salmonella. Two scenarios can be envisaged in antimicrobial resistance: 1) dissemination of an antimicrobial-resistant clone or 2) dissemination of a resistant gene carried in a genetic element. Integrons have also been considered as a tool for studying molecular epidemiology.

The aim of this study was to determine the antimicrobial susceptibility and the molecular mechanisms of resistance of Salmonella spp. strains causing acute gastroenteritis in Cuba and to determine the potential dissemination of a resistant clone.

MATERIALS AND METHODS

Bacterial isolates. A total of 34 sporadic Salmonella strains isolated from feces of patients with acute gastroenteritis isolated from different regions of Cuba in 2002 were analyzed. The strains were isolated by the different Provincial Reference Laboratories of each region of the country (the laboratories belong to a National Surveillance Network) and sent to the National Reference Laboratory of Cuba. The strains were further studied in the Laboratory of Clinical Microbiology at the Clínic Hospital in Barcelona, Spain. The strains were isolated and identified by conventional methods.

Serotyping and phage typing. Serotyping and phage typing were performed in the Carlos III Institute (Madrid, Spain), taking into account the Manual of Kauffman and White and following the recommendations of Edward and Ewing.

Antimicrobial susceptibility testing. The antimicrobial susceptibility test to nine antimicrobial agents, ampicillin (AMP), amoxicillin/clavulanic acid (AMC), nalidixic acid (NAL), tetracycline (TET), trimethoprim/sulphamethoxazole (SXT), chloramphenicol (CHL), gentamicin (GEM), ciprofloxacin (CIP), and spectinomycin (SPT), was performed using the Kirby-Bauer method. Interpretation of results was carried out according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines.

Detection of the mechanisms of resistance. To determine the quinolone-resistance mechanisms, mutations in the quinolone-resistant determining region (QRDR) of the gyrA gene were detected by polymerase chain reaction (PCR) and DNA sequencing. A previously described colorimetric assay was performed to detect the presence of chloramphenicol acetyl transferase activity (CAT).

The presence of the cmlA and floR genes associated with chloramphenicol resistance, the genes encoding β-lactamases (tem-like, carb-like, shv-like, and oxa 1-like), and the tet A, tet B, and tet G genes related to tetracycline resistance acquisition were detected by PCR. To
determine the mechanism of trimethoprim resistance, the presence of dihydrofolate reductases was detected by PCR with generic primers and posterior restriction fragment length polymorphism (RFLP) with the appropriate restriction enzyme according to previously described methodology.\textsuperscript{15} The PCR products were detected by electrophoresis in 2% agarose gels. All the primers and PCR conditions used in this study have been previously described.\textsuperscript{3}

**Detection of integrons.** In all the resistant *Salmonella* strains, the presence of Class 1 integrons was detected by PCR with specific primers described previously.\textsuperscript{14}

**DNA sequencing of the PCR products.** The purified PCR products visualized in gels were processed for DNA sequencing and analyzed in an automatic DNA sequencer (ABI 377; Perkin Elmer, Emeryville, CA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer).

**Low-frequency restriction analysis of chromosomal DNA and pulsed field gel electrophoresis.** The analysis of chromosomal DNA by digestion with low frequency of cleavage restriction enzymes and separation of the fragments by pulsed field gel electrophoresis (PFGE) was performed as follows. *Salmonella* strains were cultivated in brain-heart infusion (BHI) and incubated overnight. They were resuspended in buffer TE-1. The plugs containing the DNA were elaborated with agarose insert 1.8%.\textsuperscript{16} Chromosomal DNA contained in the agarose plugs was digested with *XbaI* (Roche Diagnostic, Mannheim, Germany), and PFGE was performed with the CHEF DR II system (Biorad Laboratories, Hercules, CA) in 0.5× Tris-Borate-EDTA buffer (Severn Biotech, Kidderminster, UK). DNA restriction fragments were resolved in agarose (Bio-Rad) 1% wt/vol gels, and a low-range PFGE marker (New England Biolabs, Beverly, MA) was used as a size standard. Electrophoresis conditions were of 2.0–64 seconds for 20 hours.\textsuperscript{17}

**RESULTS**

Of the 34 *Salmonella* strains studied from different regions around the country, *Salmonella typhimurium* was the most frequent, with 15 strains (44%), 13 of which belonged to phagotype 104, with 2 strains showing a non-recognized phagotype pattern. *Salmonella agona* was the second most frequently isolated serotype with eight strains (23%), followed by *Salmonella hadar* with two strains. Only one strain was found in other serotypes, such as *Salmonella senftenberg, Salmonella infantis, Salmonella brandenburg, Salmonella orion, Salmonella saintpaul*, and one monophasic. Three strains were non-typable.

In the 34 *Salmonella* strains studied, 22 strains presented resistance to at least one antimicrobial agent, with *S. typhimurium* with 15 strains (68%) being the most prevalent among the resistant *Salmonella*. High levels of resistance were found mainly to tetracycline in 18 strains (53%), spectinomycin in 17 strains (50%), amoxicillin in 15 strains (44%), and to chloramphenicol in 14 strains (41%). In addition, three strains presented resistance to amoxicillin/clavulanic acid, and eight strains (24%) showed intermediate resistance to amoxicillin/clavulanic acid (Table 1).

Molecular epidemiologic analysis was performed in *S. typhimurium* and *S. hadar* because of their multiresistance and in *S. agona* because of the fact that it was the second most prevalent serotype isolated. *S. typhimurium* showed five patterns of PFGE, being the pattern number 1 the most frequent with 11 isolates of 15 (Table 2; Figure 1). Although showing a different phagotype, the two strains corresponding to serotype Hadar presented an identical pattern of PFGE (Table 2). Among the eight *Salmonella* strains belonging to serotype Agona, six of the seven susceptible isolates belonged to the same PFGE pattern, whereas the remaining susceptible isolate and the resistant isolate each showed a different pattern. The geographical distribution of the resistant clones is shown in Table 2, which shows that the main *S. typhimurium* clone (clone 1) was detected in eight different provinces, distributed throughout the island, and the *S. agona* clone was disseminated in two provinces: Cienfuegos and Holguín.

Analysis of the molecular mechanisms of resistance showed that resistance to tetracycline was caused by the presence of the *tet G* gene (13 of 18 tetracycline-resistant strains) and the *tet A* gene (6 of 18 tetracycline-resistant strains). One strain presented both resistant determinants. The main mechanism of ampicillin resistance was the presence of CARB type β-lactamases, observed in 13 of 15 ampicillin-resistant strains, with tert of PFGE, being the pattern number 1 the most frequent with 11 isolates of 15 (Table 2; Figure 1). Although showing a different phagotype, the two strains corresponding to serotype Hadar presented an identical pattern of PFGE (Table 2). Among the eight *Salmonella* strains belonging to serotype Agona, six of the seven susceptible isolates belonged to the same PFGE pattern, whereas the remaining susceptible isolate and the resistant isolate each showed a different pattern. The geographical distribution of the resistant clones is shown in Table 2, which shows that the main *S. typhimurium* clone (clone 1) was detected in eight different provinces, distributed throughout the island, and the *S. agona* clone was disseminated in two provinces: Cienfuegos and Holguín.

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only one strain of S. hadar showing a TEM type β-lactamases. No strain presented OXA type and SHV type β-lactamases. All chloramphenicol-resistant isolates presented the floR gene, and only the S. senftenberg strain showed CAT activity. None of the isolates presented the cmlA gene. One strain presented resistance to trimethoprim/sulphamethoxazole because of the presence of the dfrA1 gene (Table 3). The strain with intermediate susceptibility to nalidixic acid did not show any mutation in the gyr A or par C genes.

### Table 3

Mechanisms of resistance of the resistant *Salmonella* spp.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Mechanism of resistance</th>
<th>Composition</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. hadar</td>
<td></td>
<td>temp</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>1818</td>
<td>4, 12</td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>1320</td>
<td></td>
<td>temp</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>1829</td>
<td>4, 12</td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>1837</td>
<td>S. typhimurium</td>
<td>temp</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>2667</td>
<td></td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>2673</td>
<td>S. typhimurium</td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>2834</td>
<td>S. typhimurium</td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
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<td>S. typhimurium</td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td></td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>1989b</td>
<td>4, 12</td>
<td>temp</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>1708</td>
<td></td>
<td>CAT</td>
<td>dfrA1</td>
<td></td>
</tr>
<tr>
<td>1958</td>
<td>S. typhimurium</td>
<td>temp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1822</td>
<td></td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>1270</td>
<td>S. typhimurium</td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>2821</td>
<td>S. hadar</td>
<td>temp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>885</td>
<td>S. typhimurium</td>
<td>aadA2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>410</td>
<td></td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>2841</td>
<td>S. typhimurium</td>
<td>floR</td>
<td>carb2</td>
<td></td>
</tr>
<tr>
<td>1946</td>
<td>S. typhimurium</td>
<td>aadA2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3147</td>
<td>S. typhimurium</td>
<td>aadA2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1764</td>
<td>S. agona</td>
<td>aadA2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined; SPT, spectinomycin; CHL, chloramphenicol; AMP, ampicillin; TET, tetracycline; SXT, trimethoprim/sulphamethoxazole.

Two Class 1 integrons were present in 11 of the 22 resistant strains (Table 4): one integron of 1,200 bp containing the carb2 gene, and the other with a size of approximately 1,000 bp containing the aadA2 gene. Another Class 1 integron (1,500 bp) was detected in the S. senftenberg strain and contained the aadB and aadA2 genes. Finally, two S. typhimurium and one S. agona with resistance to spectinomycin showed one Class 1 integron with a size of 1,000 bp containing the aadA2 gene.

### DISCUSSION

Although *S. typhimurium* definitive phage type (DT) 104 has been detected in human infections in England and Wales since the early 1960s, multidrug-resistant strains of this phage type were not identified until the early 1980s.18 In this study, despite only 34 Salmonella strains analyzed, we could detect the dissemination of *S. typhimurium* DT 104 in different provinces of Cuba. In addition, isolates belonging to the same clone were detected in the same geographical area, suggesting a local outbreak. The same scenario was reported in previous studies performed in Canada, United States, and Europe.19–21 This phage type is commonly resistant to ampicillin, chloramphenicol/flufenisal, spectinomycin/sulfonamides, and tetracyclines. In addition of this typical resistance pattern, some strains of *S. typhimurium* DT 104 also presented resistance to fluoroquinolones and trimethoprim. The high percentage of intermediate resistance to amoxicillin/clavulanic acid found among our strains is worthy of mention. Different reports have shown a decrease in the susceptibility of *S. typhimurium* and other serotypes to this antimicrobial agent. Espinase et al.22 reported a decreased susceptibility to amoxicillin/clavulanic acid in three *S. typhimurium* strains isolated from patients in Rumania. Similarly, in a French university hospital, many *S. typhimurium* strains with reduced susceptibility to amoxicillin/clavulanic acid were isolated.15 This fact has also been shown in *S. hadar* and *S. bilis*.23,24 Theoretically, several mechanisms may explain this reduced susceptibility to amoxicillin/clavulanic acid, including a decrease in permeability to β-lactams, as is known for *Escherichia coli*, or the over-expression of specific β-lactamases.25 In our study, all strains with reduced susceptibility carried the β-lactamases type CARB, whose over-expression may explain the origin of this reduced susceptibility to amoxicillin/clavulanic acid.

The PFGE patterns generated with the *XbaI* enzyme found in our *S. typhimurium* DT 104 strain were similar to other described before by several authors using the same conditions.19,20,27 Baggesen et al.19 compared the PFGE profile of Danish *S. typhimurium* DT 104 strains with the profile of PFGE obtained in strains isolated in five different countries

### Table 4

Class 1 integrons observed

<table>
<thead>
<tr>
<th>Strains</th>
<th>Serotype</th>
<th>Composition</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>S. typhimurium</td>
<td>carb2/aadA2</td>
<td>1,200, 1,000 bp</td>
</tr>
<tr>
<td>2</td>
<td>S. typhimurium</td>
<td>aadA2</td>
<td>1,000 bp</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>carb2/aadA2</td>
<td>1,200, 1,000 bp</td>
</tr>
<tr>
<td>1</td>
<td>S. agona</td>
<td>aadA2</td>
<td>1,000 bp</td>
</tr>
<tr>
<td>1</td>
<td>S. senftenberg</td>
<td>aadB, aadA2</td>
<td>1,500 bp</td>
</tr>
</tbody>
</table>
and found highly homogeneous profiles, indicating that the multidrug-resistant *S. typhimurium* DT 104 had probably been spread clonally in these countries.

All the strains analyzed belonging to *S. typhimurium* DT 104 presented the *floR* and *tet G* genes, being responsible for resistance to chloramphenicol and tetracycline, respectively. These genes have been previously described to be located in the *Salmonella* genomic island 1 (SGI)\(^{26,28}\). In this island, resistance genes are clustered and are bracketed by two integron structures,\(^ {18}\) identical to those found in our study. The first integron carried the *aadA2* gene, which confers resistance to spectinomycin and streptomycin, and the second integron contained the *carb2* gene, conferring resistance to ampicillin.\(^ {28}\) All the *S. typhimurium* DT 104 strains but two analyzed in our study presented these two integrons. The two strains presenting only the integron carrying the *aadA2* gene may have a truncated resistance island. Therefore, *S. typhimurium* strains with the same pattern by PFGE can present different integrons, suggesting that this is not a discriminate tool to be used in epidemiological analysis, as has been suggested.\(^ {11}\)

Three strains were not typable using the traditional serotyping techniques. Two of these three strains presented a similar mechanism of resistance profile as *S. typhimurium* DT 104. Other serotypes that showing a multiresistant phenotype were *S. hadar* and *S. senftenberg*, the latter of which is not frequently found. However, in some countries such as Brazil, it is a frequent serotype, occupying the third place of *Salmonella* isolates (10.3%) behind *S. typhimurium* and *S. enteritidis*.\(^ {27}\) According to the network of human *Salmonella* surveillances in Europe, *S. hadar* is widely distributed across the European continent.\(^ {24}\) Crucaga et al.\(^ {30}\) reported high levels of resistance, mainly to spectinomycin, ampicillin, tetracycline, and nalidixic acid, in strains of *S. hadar* isolates from humans and food, respectively. Analysis of PFGE profiles of the two resistant *S. hadar* was similar, but the PFGE in this serotype is of limited value, and the combination of different epidemiologic methods is recommended depending of the phage type studied.\(^ {23}\) In our study, these two strains belonged to different phage types and also showed different resistance phenotypes. Therefore, they may be considered two different clones, although those differences in the resistant phenotypes and phagotypes might reflect the geographical distribution of a single clonal type.

Epidemiologic analysis by PFGE of the *S. agona* strains showed a predominant clone disseminated mainly in two separate provinces: Cienfuegos and Holguín. In fact, although no molecular epidemiologic analysis have been performed, previous reports described this serotype among one of the main *Salmonella* serotypes isolated throughout the country and in several countries of American continent.\(^ {4,31–33}\) In 1998 in the United States, a total of 11 states reported an increase in cases of *S. agona*.\(^ {31}\) In Argentina and Mexico, several authors reported this serotype among the most frequent *Salmonella* isolated.\(^ {22,23}\) The presence of antimicrobial-resistant strains of this serotype has previously been reported.\(^ {34}\) However, the prevalence of antimicrobial-resistant strains is lower than the antimicrobial resistance observed in *S. typhimurium*. In some *S. agona*-resistant strains, the same resistant island, SGII, has been shown.\(^ {34}\) However, in our study, the multidrug-resistant *S. agona* strain did not show the resistant determinants located in this island, although the integron, carrying the *aadA2* gene, was found.

Our results show the dissemination of a multiresistant clone of *S. typhimurium* DT 104 and an antimicrobial susceptible clone of *S. agona* in two separate regions in Cuba. This work presents the first description in Cuba of the multiresistant clone of *S. typhimurium* DT 104 since first being identified in the United Kingdom in the late 1980s.\(^ {18}\)

Received October 6, 2005. Accepted for publication December 1, 2005.

Financial support: This study was funded by grant FIS02/0353 from the Spanish Ministry of Health, by grant 2005 SGR 00444 from the Departament d’Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya, Spain. R. Cabrera has a fellowship from Fundación Carolina and BBVA, Spain, and J. Ruiz has a fellowship from RICET.

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