Characterization of a Laboratory-Derived, High-Level Ampicillin-Resistant *Salmonella enterica* Serovar Typhimurium Strain That Caused Meningitis in an Infant

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Received 16 July 2001/Returned for modification 12 November 2001/Accepted 11 February 2002

A *Salmonella enterica* serovar Typhimurium strain that harbored a plasmid carrying a TEM-1-type β-lactamase gene was isolated from the blood and cerebrospinal fluid of an infant with meningitis. This 3.2-kb plasmid was further characterized to be a nonconjugative pGEM series cloning vector containing a foreign insert. The strain was likely laboratory derived and contaminated the environment before it caused the infection.

*Salmonella*, a genus belonging to the family *Enterobacteriaceae*, comprises a large and complex group of human pathogens that have long been associated with a wide spectrum of infectious diseases, including typhoid fever and nontyphoidal salmonellosis. Enterocolitis caused by nontyphoidal *Salmonella* is usually self-limiting, and antimicrobial treatment is seldom required (10). Nevertheless, effective antimicrobial treatment is essential if the infection spreads beyond the intestine.

Extraintestinal *Salmonella* infections must be treated with ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol, or extended-spectrum cephalosporins. About 10 to 20% of nontyphoidal *Salmonella* isolates in the United States are resistant to ampicillin, and the rate is even higher in developing countries. Nontyphoidal salmonellosis has been rampant in Taiwan (2, 3). In Chang Gung Memorial Hospital (CGMH), a 3,500-bed university-affiliated medical center located in northeastern Taiwan, a total of 7,986 salmonella isolates were isolated between 1983 and 1999. Serogroup B has been the most prevalent. The proportion of serogroup B isolates to the total salmonella isolates was stationary around 60 to 70% (average, 66.8%) through these years. Most of the serogroup B isolates belonged to serovar Typhimurium (2–4).

For serogroup B isolates, the resistance to ampicillin has increased from 50 to 80% after 1988 in our hospital. During a 16-month period between 1994 and 1995, we checked the plasmid profiles of 224 clinical isolates of *Salmonella enterica* serovar Typhimurium by the method of Kado and Liu (5). The majority of these isolates contained a large plasmid of 90 to 100 kb in size; furthermore, 84% of these large plasmids were virulence plasmids (pSTV) (4). Only 5 strains contained an 11-kb small plasmid, which turned out to be an R factor encoding tetracycline resistance (4). The relationship of these plasmids and antimicrobial resistance was not checked further. However, a recent study by Llunes et al. (6) demonstrated that nearly all of the virulence plasmids of *S. enterica* serovar Typhimurium carried genes coding for PSE-1 or TEM-1 β-lactamase.

A 3-month-old infant living in northern Taiwan presenting with meningitis was admitted to CGMH in May 1999. The patient’s clinical course was complicated with a subdural empyema, which necessitated surgical drainage in addition to antimicrobial therapy. The blood as well as cerebrospinal fluid cultures yielded serogroup B *Salmonella*, which was subsequently typed to be *S. enterica* serovar Typhimurium. This isolate contained a 90-kb plasmid along with a 3.2-kb small plasmid (Fig. 1A). The infant’s severe illness and the unusual plasmid profile prompted us to perform a further investigation on the isolate.

The laboratory strains of *S. enterica* serovar Typhimurium and *Escherichia coli* used in this study are shown in Table 1. Plasmid profiles were determined by the method of Kado and Liu (5). To extract plasmid DNA, a modified alkaline method was used (5, 11). The transfer of a plasmid from one strain to the other was performed either by transformation or by conjugation, as described previously (11, 12, 13). The antimicrobial susceptibilities of the bacterial strains to antimicrobial agents were determined by the broth microdilution or disk diffusion method in accordance with the standards of the National Committee for Clinical Laboratory Standards (8, 9).

The clonal relationship between individual isolates was assessed by infrequent-restriction-site PCR (IRS-PCR) as previously described (7, 14). Bacterial suspensions were digested for 2 h with 1 mg of proteinase K (Worthington Biochemical Corporation, Lakewood, N.J.)/ml at 56°C followed by treatment with sonication for 40 min. The crude DNA was further digested by *Xba*I and *Hha*I, and the subsequent ligation and amplification were performed as previously described (7, 14). The PCR products (5 μl) were loaded into the wells of a 12.5% polyacrylamide gel. After electrophoresis for 50 min at 600 V, the gel was silver stained and photographed. The gels, staining solutions, and electrophoresis-related instruments were purchased from Pharmacia Biotech (Upsala, Sweden). To ensure the reproducibility of the method, each isolate was examined twice.

Five oligonucleotide primers were used to determine the
nucleotide sequence of the small plasmid (pSTCG). The sequence of the first primer is GGAGTCAGGCAACTATGGAT, which was designed according to the published sequence of the TEM-1-type β-lactamase gene. To amplify the whole β-lactamase gene, another pair of primers was used: ATGAGTATTCAACATTTCCGTGT (forward) and TTACCAATGCCTAATCAGTGAGG (reverse). Since the preliminary sequence data obtained by using the first primer suggested that the plasmid was likely a commercial cloning vector, we further used the M13/pUC sequencing primers (forward and reverse) (MBI Fermentas Inc.) to amplify and sequence the multiple cloning site region of the plasmid. Sequencing was performed on an ABI 373A automatic sequencer (Perkin-Elmer, Applied Biosystems). The sequences were analyzed with Pcgene software (Intellegenetics). The search for homologous sequences was done in the GenBank database with the FASTA software through the internet.

The isolate from the child (CGST1) showed resistance to ampicillin and chloramphenicol (Table 1). The MIC of ampicillin was extremely high at ≥64 μg/ml. This isolate contained a 90-kb large plasmid and a 3.2-kb small plasmid, pSTCG (Fig. 1A). A PCR analysis confirmed that this isolate was spvC positive, indicating that the large plasmid was a virulence plasmid (1). When pSTCG was extracted and transformed into E. coli HB101 by using ampicillin resistance as the selection marker, the transformant remained susceptible to chloramphenicol. This result suggested that pSTCG carried an ampicillin resistance gene, whereas chloramphenicol resistance was chromosome mediated. This was also the case when pSTCG was transformed into the three laboratory strains of S. enterica

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>MIC (μg/ml) of:</th>
<th>Susceptibility to:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>AMP</td>
<td>CRO</td>
</tr>
<tr>
<td>S. enterica serovar Typhimurium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGST1 (clinical isolate)</td>
<td>pSTV, pSTCG</td>
<td>≥64</td>
<td>0.25</td>
</tr>
<tr>
<td>C5</td>
<td>pSTV</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>CNP505</td>
<td>2</td>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>CNP101</td>
<td>2</td>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>LBNP4417</td>
<td>2</td>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>C5/pSTCG</td>
<td>≥64</td>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>CNP101/pSTCG</td>
<td>≥64</td>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>LBNP4417/pSTCG</td>
<td>≥64</td>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
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<tr>
<td>HB101</td>
<td>pSTCG</td>
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<td>HB101/pSTCG</td>
<td>≥64</td>
<td>0.25</td>
<td>S</td>
</tr>
<tr>
<td>JC1569</td>
<td>F::Tn10 lac&quot; (Ts)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviations: AMP, ampicillin; CRO, ceftriaxone; SXT, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; S, susceptible; R, resistant; ND, not done.
serovar Typhimurium, C5, CNP101, and LBNP4417. Interestingly, the copy numbers of pSTCG in E. coli HB101 and the two plasmidless strains of S. enterica serovar Typhimurium, CNP101 and LBNP4417, were high; on the other hand, in C5 or the original isolate, the copy number was only about one-half of that in the three other strains (Fig. 1A). Nevertheless, the expression level of ampicillin resistance is copy number independent; all the strains that carried pSTCG showed the same level of resistance to ampicillin (MIC ≈ 64 μg/ml).

pSTCG was then transformed into JC1569, an E. coli strain carrying an F plasmid. However, we were unable to transfer pSTCG from JC1569/pSTCG to either C5 or LBNP4417 by mating when using both ampicillin and tetracycline resistance as the selection markers. On the other hand, F’::Tn10 lac’ (Ts) was successfully transferred into C5 and LBNP4417 when tetracycline resistance alone was used as the selection marker. The transconjugant, which appeared as red colonies (Lac+) on MacConkey agar, contained F’::Tn10 lac’ (Ts) but no pSTCG.

To further characterize the plasmid as well as its β-lactamase gene, PCR sequencing was performed, and a 682-bp DNA sequence was first obtained. The sequence was found to be 100% homologous to a DNA fragment of a pGEM series plasmid, which is a commercially used cloning vector. The DNA fragment actually included partial sequences of bla and fl ori genes, the latter of which is unique in pGEM series vectors (GenBank/EMBL accession number X65317). The bla sequence obtained by this as well as another pair of primers was shown to be nearly identical to that of the ampicillin resistance gene of pBR322, suggesting that this is a TEM-1-type bla gene. By using two more primers, we finally were able to obtain a 2,573-bp sequence of pSTCG. The search for homologous sequences confirmed that this 2,573-bp fragment contained sequences identical to the published sequences of the pGEM-3Zf(−) vector (positions 1265 to 3197 and 0 to 640; GenBank/EMBL accession number X65307), with a foreign insert cloned between the PstI and KpnI cloning sites. This DNA insert contained some sequences homologous to the xylene oxygenase promoter of pSPZ3.

pGEM series vectors have never been used in any laboratories of our institute. All of the bacterial strains, including those that were constructed, were further checked by IRS-PCR. As shown in Fig. 1B, all S. enterica serovar Typhimurium strains showed similar PCR patterns; however, the clinical isolate of the child had at least two bands that differed from those of the laboratory strains.

Our findings confirmed that pSTCG, which conferred ampicillin resistance to an S. enterica serovar Typhimurium strain, was in fact an in vitro-engineered, commercially available cloning vector. pSTCG, a pGEM vector with a foreign insert, is nonconjugative, and not mobilized by the F plasmid, indicating that this plasmid was previously transferred to S. enterica serovar Typhimurium by artificial methods, probably in a research laboratory. It is possible that this strain was disseminated to the community and caused the infection by contaminating the environment or water source.

Our findings also demonstrated that the presence of an indigenous virulence plasmid may affect the copy number of a foreign R plasmid in S. enterica serovar Typhimurium while the expression of an antibiotic resistance gene of the R plasmid remained unchanged. The mechanism of this interaction deserves further study. Earlier reports have shown that S. enterica serovar Typhimurium may acquire the blaTEM-1 gene through the acquisition of the transferable R plasmid (6). The blaTEM-1 gene identified in Salmonella is met with increasing frequency in other gram-negative enteric species, such as E. coli, suggesting that S. enterica serovar Typhimurium may inherit the gene from other bacterial sources. Most of the S. enterica serovar Typhimurium clinical isolates carried a virulence plasmid (4). It appears that the virulence plasmid of S. enterica serovar Typhimurium and pGEM, or other R plasmids carrying blaTEM-1, belong to different incompatibility groups. This observation supports the speculation of a dissemination of blaTEM-1 between Salmonella and other gram-negative enteric bacteria.

Our findings provide evidence that a laboratory-derived, ampicillin-resistant S. enterica serovar Typhimurium strain caused meningitis in an infant. Appropriate and safe processing of biological waste derived from hospitals and research laboratories can never be overemphasized.

This work was supported by grant CMRP876 from CGMH.

REFERENCES


