Molecular Epidemiology of Ampicillin-Resistant Clinical Isolates of Salmonella enteritidis

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During the last 6 years in Greece, there has been a significant increase in the number of ampicillin-resistant Salmonella clinical isolates reported. In this study 23 ampicillin-resistant Salmonella strains, consecutively isolated from patients with epidemiologically unrelated cases of food poisoning, were investigated. By serotyping and phage typing, 21 of these strains were identified as Salmonella enteritidis phase type 6a, 1 was identified as Salmonella typhimurium, and 1 was identified as Salmonella saintpaul. By plasmid pattern analysis, the 21 S. enteritidis strains were further differentiated into five groups. Group I consisted of 5 strains (carrying two plasmids of ca. 38 and 34 MDa), group II consisted of 10 strains (three plasmids of ca. 38, 34, and 2.5 MDa), group III consisted of 3 strains (four plasmids of ca. 38, 34, 15, and 2.5 MDa), group IV consisted of 1 strain (five plasmids of ca. 100, 38, 34, 24, and 15 MDa), and group V consisted of 2 strains (three plasmids of ca. 100, 38, and 24 MDa). Ampicillin resistance was easily transferred to Escherichia coli and was associated with the transfer of the 34-MDa plasmid, classified in the N incompatibility group for all strains of groups I to IV, and with the transfer of the 100-MDa plasmid for the group V strains. EcoRI restriction endonuclease digestions showed an extensive homology among the 34-MDa conjugative R plasmids. Hybridizations of the EcoRI restriction fragments of the 34-MDa plasmids with a TEM-type probe revealed the locus of the β-lactamase gene to be situated on a ca. 6.6-MDa fragment, common in all plasmids. These results indicate that ampicillin resistance in Greece is due to the spread of a limited number of clones of S. enteritidis phase type 6a, carrying related 34-MDa R plasmids. Work is in progress to obtain a better understanding of ampicillin resistance in S. enteritidis.

Nontyphoid salmonellas are recognized as important causes of food poisoning worldwide (2, 5). Although the resulting gastrointestinal illness does not usually require antibiotic therapy, antibiotic resistance among the causative agents is a public health concern, as these infections may be complicated with septicemia, endocarditis (10), empyema (3), meningitis (7), and bone and joint infections (19), especially in immunocompromised individuals. Salmonella infection appears to be one of the most typical examples of an enteric disease implicated mainly in common-source epidemics. Strain identification is essential for the effective investigation of these outbreaks, and traditional as well as molecular typing methods have been successfully applied (28). In that respect, several studies have demonstrated that plasmid profile analysis could be a reliable epidemiological tool for the differentiation of epidemic and nonepidemic strains, and for the elucidation of the epidemiology of these foodborne pathogens (26, 29).

In Greece, a relative increase in the frequency of isolation of Salmonella enteritidis from humans from 30% of the total Salmonella sp. isolations in 1987 to about 82% in 1991 has been observed (20). Moreover, a sharp increase in the rate of isolation of ampicillin-resistant Salmonella spp. from 7.9% in 1987 to 30.4% in 1991 (Table 1) has also been noticed.

The purpose of this study was to investigate the molecular epidemiology of these infections. Serotyping, phage typing, antimicrobial susceptibility testing, and plasmid pattern analysis were used for this purpose. For a better characterization of plasmids, DNA-DNA hybridizations were also performed.

MATERIALS AND METHODS

Strains. Twenty-three ampicillin-resistant Salmonella strains, consecutively isolated from outpatients with gastrointestinal illness admitted to two hospitals in Athens, Greece (Hippocration General Hospital and Penteli Children’s Hospital), from the beginning of June to the end of August 1991, were analyzed. All isolates were epidemiologically unrelated and represented sporadic cases of food poisoning. They were identified by the API 20E system (bioMérieux sa, Marcy-l’Etoile, France). For conjugation experiments, Escherichia coli K-12 strains R176 (Str“), 20R764 (Rif“), 26R793 (Rif“), and 14R525 (Nal“) were used as recipients. E. coli 39R861, which harbors plasmids of 98, 42, 23.9, and 4.6 Mda, was used for estimation of plasmid size (30).

Susceptibility testing. Antibiotic susceptibility testing was performed by a disc diffusion method on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, United Kingdom) according to the current recommendations of the National Committee for Clinical Laboratory Standards (17). Discs impregnated with ampicillin, kanamycin, streptomycin, chloramphenicol, tetracycline, and trimethoprim were purchased from Oxoid.

Serotyping and phage typing. All isolates were serotyped by the methods of Kaufman (15) for cell wall (O) and flagellar (H) antigen identification. Phage types were determined by use of the system described by Ward et al. (33), with 10 typing

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TABLE 1. Number of S. enteritidis isolates and percentage of ampicillin resistance during the years 1987 to 1992

<table>
<thead>
<tr>
<th>Yr of isolation</th>
<th>No. of isolates</th>
<th>Ampicillin resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1987</td>
<td>150</td>
<td>7.9</td>
</tr>
<tr>
<td>1988</td>
<td>182</td>
<td>7.5</td>
</tr>
<tr>
<td>1989</td>
<td>282</td>
<td>9.2</td>
</tr>
<tr>
<td>1990</td>
<td>409</td>
<td>24.6</td>
</tr>
<tr>
<td>1991</td>
<td>513</td>
<td>29.1</td>
</tr>
<tr>
<td>1992</td>
<td>521</td>
<td>30.4</td>
</tr>
</tbody>
</table>

* Data were derived from two large hospitals in Athens, Greece (Hippokration General Hospital and Penteli Children’s Hospital).

Phages. Phage types were identified with a series of numbers and letters which corresponded to phage lysis patterns previously reported (33).

Transfer of resistance. Conjugation experiments were carried out in broth as previously described (8). Transconjugants were selected on MacConkey agar containing ampicillin (10 μg/ml) and streptomycin (500 μg/ml), rifampin (90 μg/ml), or nalidixic acid (40 μg/ml).

Plasmid DNA analysis. Plasmid DNA was extracted by an alkaline lysis procedure described by Takahashi and Nagano (25). The isolated DNA was transferred to 0.7% agarose gels, stained with ethidium bromide, and analyzed under UV illumination by the BIO-PROFIL (Vilber Lourmat, Marne La Vallee, France) imaging analysis system.

Restriction endonuclease analysis. Plasmid DNA was extracted from the transconjugants by the method described by Olsen (18) and digested with EcoRI restriction endonuclease (Boehringer Mannheim Biochemicals, Mannheim, West Germany) according to the instructions of the manufacturer. The digests were subjected to electrophoresis through 0.8% agarose. HindIII digests of bacteriophage lambda provided linear molecular size markers.

Incompatibility grouping. Incompatibility grouping of the Salmonella plasmids was performed as previously described (8). Briefly, the transconjugants carrying the Salmonella plasmids were used as donors in conjugation experiments with a set of E. coli strains, each of which harbored a plasmid of a known incompatibility group. In general, if the plasmids belonged to the same incompatibility group, the resident plasmid was displaced. In that case, the test for compatibility was repeated in the opposite direction to find out whether the plasmid that had been eliminated in the first test could, in its turn, eliminate the other. If the plasmids belonged to different incompatibility groups, both plasmids were established in the transconjugant. In this case, the transconjugants were tested for their ability to carry both plasmids stably and separately.

Hybridization experiments. The 296-bp HindII-PstI fragment of plasmid pBR322 was used as the TEM-type β-lactamase probe (21). The vagA gene (22) cloned from the 60-MDa serovar-specific mouse virulence plasmid of Salmonella typhimurium was used as a virulence-associated probe. Colony and Southern blot hybridizations were performed with a nonradioactive DNA labeling and detection kit (Boehringer Mannheim) under high-stringency conditions as previously described (32).

RESULTS

By serotyping, 21 of the Salmonella strains studied were identified as S. enteritidis, 1 was identified as S. typhimurium, and one was identified as Salmonella saintpaul. By phage typing, all S. enteritidis strains were found to belong to phage type 6a. Plasmid pattern analysis of the 21 S. enteritidis strains permitted further differentiation of the strains into five groups (Table 2). Group I included 5 strains, carrying two plasmids of ca. 38 and 34 MDa; group II was represented by 10 strains, with three plasmids of ca. 38, 34, and 2.5 MDa; group III consisted of 3 strains, with four plasmids of ca. 38, 34, 15, and 2.5 MDa; 1 strain, carrying five plasmids of ca. 100, 38, 34, 24, and 15 MDa belonged to group IV; and 2 strains, carrying three plasmids of ca. 100, 38, and 24 MDa, were allocated to group V (Table 2). All strains were resistant solely to ampicillin, except two strains of group V which were also resistant to kanamycin and tetracycline (Table 2).

Ampicillin resistance was easily transferred at high frequency to E. coli recipients (Table 2). Agarose gel electrophoresis of the transconjugants revealed that ampicillin resistance was encoded by the ca. 34-MDa plasmid in all isolates except for the two belonging to group V, in which the resistance determinants (resistance to ampicillin, kanamycin, and tetracycline) were encoded by a ca. 100-MDa plasmid (Table 2). The study of the incompatibility of the 34-MDa plasmids classified them in the N incompatibility group.

Plasmid fingerprinting by cleavage patterns, generated after digestion with EcoRI restriction endonuclease, showed extensive homology among the 34-MDa conjugal R plasmids. The two 100-MDa plasmids of group V also demonstrated extensive homology. The fragmentation patterns of the 34- and 100-MDa R plasmids were different. However, at least three common fragments (ca. 10.9, 8.5, and 6.6 MDa) between the 100- and the 34-MDa plasmids were evident (Fig. 1).

Hybridizations of Southern blots of the plasmids showed that the 38-MDa plasmid encoded the vagA gene (data not shown). In other experiments, colony hybridization with the TEM-type probe gave positive signals for both clinical isolates and transconjugants. Hybridizations of the EcoRI restriction fragments of the 34- and 100-MDa plasmids with the TEM-type probe revealed the locus of the β-lactamase gene to be on a ca. 6.6-MDa fragment, common in all 34- and 100-MDa plasmids. In addition, a positive signal was also evident on a ca. 16.4-MDa fragment of the 100-MDa plasmid (Fig. 2).

TABLE 2. Plasmid pattern analysis and resistance phenotype of S. enteritidis phage type 6a isolates

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of strains</th>
<th>Resistance phenotype</th>
<th>Plasmid pattern (MDa)</th>
<th>Resistance phenotype</th>
<th>Plasmid pattern (MDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>Amp'</td>
<td>38, 34</td>
<td>Amp'</td>
<td>34</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>Amp'</td>
<td>38, 34, 2.5</td>
<td>Amp'</td>
<td>34</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>Amp'</td>
<td>38, 34, 15, 2.5</td>
<td>Amp'</td>
<td>34</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>Amp'</td>
<td>100, 38, 34, 24, 15</td>
<td>Amp'</td>
<td>34</td>
</tr>
<tr>
<td>V</td>
<td>2</td>
<td>Amp' Kan' Tet'</td>
<td>100, 38, 24</td>
<td>Amp' Kan' Tet'</td>
<td>100</td>
</tr>
</tbody>
</table>

* Amp', Kan', and Tet', resistance to ampicillin, kanamycin, and tetracycline, respectively.
DISCUSSION

All *S. enteritidis* strains examined contained a plasmid of ca. 38 MDa which hybridized with the virulence-associated gene (vagA) probe. This finding is in agreement with previous data on the 38-MDa plasmid of *S. enteritidis* (12, 16), which is nontransferable in vitro and has not undergone detectable molecular evolution under the influence of antibiotic pressure (14). The precise phenotype of this plasmid is best described as virulent for BALB/c mice. However, its role in human infections is questionable, since human gastroenteritis isolates lacking any plasmid or chromosomal virulence-associated gene sequences have been described (6).

Over the last 10 years, this microorganism has been reported with greatly increased frequency worldwide: the World Health Organization *Salmonella* surveillance data obtained from 1979 to 1987 indicated an international increase in the relative frequency of isolation of *S. enteritidis*, which was found to be the most common serotype in eight European countries in 1987 (24). Recent reports from England (1) and Spain (23) also show *S. enteritidis* as the most frequently isolated serotype, with incidences of 60 and 86%, respectively. Similarly, in the United States between 1979 and 1989, *S. enteritidis* infections increased sixfold in the northeastern and mid-Atlantic regions (4). Data from the Greek Salmonella Reference Laboratory, Athens School of Hygiene, presented an upsurge in the relative frequency of isolation of *S. enteritidis* from 25% in 1987 to 82% in 1991 (20). Investigations in individual countries (24, 27) suggest that the reason for the global increase in *S. enteritidis* isolation is related to the consumption of eggs and poultry which harbor the organism. Most of the *S. enteritidis* infections prior to 1990 were caused by phage type 4 (20, 34). The fact that most strains of this phage type are antibiotic sensitive explains the low incidence of resistance in *S. enteritidis* during the years previous to 1990.

Antibiotic resistance is a serious problem in Greece, and high rates of resistance to antibiotics are observed among isolates of *Enterobacteriaceae* in Greek hospitals (31, 32). More specifically, 49.8% of *E. coli* clinical isolates obtained at Hippocrates General Hospital and 34.9% of *E. coli* clinical isolates obtained at Penteli Children's Hospital in 1990 were ampicillin resistant.

The high rate of ampicillin resistance in *S. enteritidis* observed during the last 6 years in Greece seems to be due to the appearance and spread of strains belonging to phage type 6a. Phage type 6a was very rarely encountered in Greece before 1987, with a frequency of isolation of less than 2% (20). All *S. enteritidis* phage type 6a strains tested were ampicillin resistant.

Data on the antibiotic susceptibility of this phage type from other countries could not be found in the available literature, although it is noted that the Ward phage type 6a reference strain is ampicillin sensitive (33). Ampicillin resistance was found to be due to a TEM-type β-lactamase, encoded mostly on 34-MDa conjugative plasmids and in a minority of cases on 100-MDa plasmids. It could be postulated that such ampicillin-associated plasmids, common in all ampicillin-resistant isolates, might be responsible for the observed resistance-phage type relationship, as has already been shown for the *S. enteritidis* phage type 24 resistance to ampicillin, streptomycin, and tetracycline (9).

The genetic relatedness of the 34-MDa plasmids in most of the *S. enteritidis* strains examined demonstrates the wide distribution of these plasmids in our geographic area. The observation that the 100-MDa plasmid shares common fragments with the 34-MDa plasmids upon EcoRI restriction enzyme digestion and the fact that the TEM-type probe hybridized on a 6.6-MDa fragment in both 100- and 34-MDa plasmids are consistent with the hypothesis of a possible common evolution of these two *S. enteritidis* plasmids.

It would be expected that in cases where epidemiological association with a common exposure was absent, ampicillin-resistant *S. enteritidis* patterns would prove to be different. Although phage typing is essential for the primary subdivision of serovars, this approach would not prove useful with our *S. enteritidis* ampicillin-resistant isolates, which are characterized by only one phage type (phage type 6a). For a more complete epidemiological identification of those strains, genotypic strain differentiation was necessary. In this respect, agarose gel electrophoresis of plasmid DNA permitted the division of the 21 strains into five groups. Except for the serotype-specific virulence-associated plasmid (38 MDa) and the conjugative ampicillin-resistance-mediating plasmids (34 and 100 MDa), the plasmids are cryptic, nontransferable, and therefore more
valuable for the allocation of the respective isolates into distinct clones. Our results are in line with previous reports indicating the existence of multiple plasmid profiles within one phage type (23). The ultimate source of the Salmonella R plasmids found during this study cannot be determined. Normal gut flora of humans and animals may be suspected as a pool of plasmids mediating ampicillin resistance. Because of the complexity of sequence events, it is generally difficult to document the colonization of antibiotic-resistant microorganisms of animal origin in humans and the production of disease (13). Although accumulated data trace multiple-drug-resistant Salmonella strains to animals (34), other reports have shown Salmonella isolates from human sources to be more resistant than those from other sources and have shown that multiple drug resistance occurred only among human isolates (11). The obscurity in the transition process is also reflected in the controversy over the addition of antimicrobial agents to animal feed. However, it is certain that antibiotic resistance can emerge and be selected as a result of subtherapeutic use of antimicrobial agents for animals and that such antibiotic-resistant Salmonella strains can cause serious human disease, especially in persons being administered antibiotics (13). If the 34-MDa plasmids had circulated separately in human and animal flora, their structures might be expected to have diverged. Figure 1 indicates that this has not occurred.

The evolution of a new phage type of S. enteritidis in Greece, in connection with the spread of similar ca. 34-MDa ampicillin-resistant plasmids of incompatibility group N into a few clones of phage type 6a, seems to be the main cause of the increase of ampicillin resistance in S. enteritidis in Greece. Moreover it could be suggested that isolates of a certain S. enteritidis phylogenetic lineage expressed by susceptibility to phage 6a are more likely to develop ampicillin resistance through the acquisition of certain R plasmids. Work is in progress to further elucidate the origin of the R plasmids in Salmonella spp.

REFERENCES