Sulphonamide resistance gene sul3 found in Escherichia coli isolates from human sources

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Objectives: The aim of this study was to investigate the molecular basis of an observed increasing resistance to trimethoprim and sulphonamides despite a simultaneous decline in co-trimoxazole consumption. The distribution of sulphonamide resistance genes sul1, sul2 and the recently discovered sul3 was studied in a collection of clinical isolates of Enterobacteriaceae.

Methods: PCR with primers specific for sul1, sul2 and sul3 was used to detect the three known sulphonamide resistance genes in the isolate collection. Sequence analysis was used for confirmation of results. Restriction endonuclease digestion and conjugational transfer assays were used for plasmid analysis.

Results: In 64 sulphonamide-resistant isolates, 39 sul1 genes and 48 sul2 genes were detected. Twenty-five isolates carried both sul1 and sul2 and two were negative for both genes. With PCR and sequence analysis these two were shown to harbour the new sulphonamide resistance gene sul3, which was carried by different plasmids.

Conclusions: Sulphonamide resistance gene sul3, which is widespread among pigs in Switzerland, has now also been identified in two different clinical isolates of Escherichia coli, located in urinary tract infections in patients in Sweden.

Keywords: urinary tract infections, PCR, plasmids

Introduction

Sulfamethoxazole in combination with trimethoprim (co-trimoxazole) remains an antimicrobial alternative in the treatment of several infectious diseases and is, according to the WHO, the drug of choice for some conditions.

Clinically relevant sulphonamide resistance among Gram-negative enteric bacteria is plasmid-borne and mediated by alternative sulphonamide-resistant dihydropteroate synthases. For a long time the only two known plasmid-borne genes conferring resistance to sulphonamides were sul1 and sul2.1,2 In the 1960s, these enzymes were identified and confirmed as transferred by plasmids.3,4 sul1 and sul2 are present in many different species and previously have been shown to be distributed equally among resistant isolates.2 Recently, the spread of sul2 seems to have increased, as the gene was reported to be more widespread among clinical isolates of Escherichia coli than sul1 in Denmark and the UK.1,3 In the British study, half of the observed increase was due to the acquisition of sul2-containing plasmids by isolates already carrying sul1.1 Thus it seems that many bacteria, including human pathogens, readily incorporate additional sulphonamide resistance elements.

During 1988–1999, we noted a continuous increase in co-trimoxazole resistance among E. coli isolates in the Stockholm area, despite the declining consumption of co-trimoxazole during the same period.5 We therefore investigated the molecular basis for trimethoprim and sulphonamide resistance in Gram-negative pathogens from the Karolinska Hospital area in Stockholm. Here, the presence of the sul3 gene—originally reported in E. coli from pigs in Switzerland—is described for the first time in clinical isolates from humans.7

Materials and methods

Bacterial strains

One hundred and five clinical urinary isolates, partly selected for trimethoprim resistance, were collected at the Department of Clinical Microbiology, Karolinska Hospital, during May–June 2001. Isolate species were determined by standard laboratory methods; the majority (75 isolates) were E. coli. A disc diffusion method, using Iso-Sensitest
**sul3 in E. coli clinical isolates**

Table 1. Collection of *Enterobacteriaceae* tested with PCR for carriage of sulphonamide resistance genes

<table>
<thead>
<tr>
<th>Species</th>
<th>sul1 only</th>
<th>sul2 only</th>
<th>sul1 and sul2</th>
<th>sul3 only</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>11</td>
<td>20</td>
<td>25</td>
<td>2</td>
<td>58</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>14</strong></td>
<td><strong>23</strong></td>
<td><strong>25</strong></td>
<td><strong>2</strong></td>
<td><strong>64</strong></td>
</tr>
</tbody>
</table>

Agar and antibiotic discs of sulphonamides 300 µg—sulfathiazole 111 µg, sulfadiazine 78 µg and sulfamerazine 111 µg (Oxoid AB, Sollentuna, Sweden)—was used to test for resistance to sulphonamides. Zone breakpoints for susceptibility and resistance were S ≥ 17 mm and R ≤ 12 mm in accordance with NCCLS protocols.8 Extended susceptibility tests were performed on selected isolates using antibiotic discs of ampicillin 10 µg, trimethoprim 5 µg, sulphonamides 300 µg, chloramphenicol 30 µg, cefuroxime 30 µg, amoxicillin 20 µg with clavulanic acid 10 µg, gentamicin 30 µg, streptomycin 10 µg, amikacin 30 µg, netilmicin 30 µg, kanamycin 30 µg and spectinomycin 25 µg. (Oxoid AB). Zone breakpoints for these antibiotics were in accordance with the Swedish Reference Group for Antibiotics, SRGA (www.srga.org) or the NCCLS (for streptomycin, kanamycin and amoxicillin/clavulanic acid).8

PCR for detection of sulphonamide resistance genes

Boiled bacterial suspensions were used as DNA templates for the PCR reactions, which were performed with primers specific for sul1, sul2 and sul3. Primers used for sul1 and sul2 were sul1f 5′-ATG GTG ACG GTG TTC GGC ATT CTG A-3′, sul1r 5′-CTA GGC ATG ATC TAA CCC TCG GTCT T-3′, sul2f 5′-GAA TAA ATC GET CAT CAT TTT CGG-3′ and sul2r 5′-CGA ATT CTT GGC GTT TCT TCT AGC-3′, at an annealing temperature of 64°C. For sul3, primers sul3f 5′-GAG CAA GAT TTT TGG AAT CG-3′, sul3r 5′-CAT TCT AGA AAA CAG TCG TAG TTC G-3′ and sul3r 5′-CAT CTG CAG CTA ACC TAG GGC TTT GGA-3′ were used at an annealing temperature of 51°C.7

DNA sequence analysis of sul3

For the sequencing reactions, an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Stockholm, Sweden) was used. Sequence analysis started from the primers sul3f2 and sul3r (see above). The samples were analysed in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Additional analysis of the raw data was carried out using the Sequencher program (Gene Codes Corporation, Ann Arbor, MI, USA).

Plasmid analysis

Plasmid DNA was isolated using the Wizard plasmid miniprep kit from Promega (Scandinavian Diagnostic Services, Falkenberg, Sweden), followed by incubation with proteinase K and extraction with phenol-chloroform-isooamylocel (25:24:1) to eliminate DNase produced by the selected bacterial strains. The plasmids were analysed by restriction endonuclease analysis using *Pvu*I, *Hind*III and *Sph*I, and the fragments were separated by agarose gel electrophoresis.

Plasmid conjugation

Recipients for plasmid conjugation assays were *E. coli* K12 derivative strains resistant to both nalidixic acid and rifampicin. Conjugal transfer experiments were performed by mixing and culturing the two strains on filters on agar plates. Transconjugants were selected on Iso-Sensitest agar plates containing sulfamethoxazole 500 mg/L and either nalidixic acid 40 mg/L or rifampicin 100 mg/L.

Results

Of 105 isolates of *Enterobacteriaceae*, 64 were resistant to sulphonamides. Of these, 14 were positive in PCR for sul1 only, and 23 were positive for sul2 only. Twenty-five isolates were positive for both sul1 and sul2. Two isolates were negative for both sul1 and sul2 but positive for sul3. None of the remaining 62 resistant isolates was positive for sul3 (Table 1).

The two *E. coli* isolates positive for sul3 were from patients with no known connection to Switzerland. They were both highly resistant to sulphonamides; one was also resistant to ampicillin, piperacillin, cefuroxime, gentamicin, trimethoprim, norfloxacin, streptomycin, kanamycin, spectinomycin and chloramphenicol; the other was also resistant to trimethoprim, spectinomycin and chloramphenicol.

The presence of sul3 in these isolates was confirmed by sequence analysis. One large plasmid was isolated from each of the two strains. The restriction patterns of the two plasmids when incubated with *Pvu*I, *Hind*III and *Sph*I were not the same. The sizes of the two plasmids were in the range 50–80 kb.

In conjugation assays, sulphonamide resistance was transferred from only one of the strains, in association with a plasmid, and resistance to trimethoprim, chloramphenicol and spectinomycin was co-transferred. Confirmation of transconjugants was done by PCR specific for sul3. As a negative control the transconjugants were spread on minimal medium plates since *E. coli* K12 cannot grow without threonine and leucine.

Discussion

A new plasmid-borne sulphonamide resistance gene related to sul1 and sul2, named sul3, has been discovered.7 It was shown to be common in *E. coli* isolates from pigs in Switzerland. We have now observed the same gene in two human clinical isolates of *E. coli* from Sweden.

The continued rise in sulphonamide resistance among bacterial pathogens is disturbing. It is interesting that new genes mediating resistance to ‘old’ antibiotics, such as sulphonamides, are still being discovered. The distribution of the sul3 gene among human bacterial pathogens has not been investigated. In earlier studies of the distribution of sul1 and sul2 in 203 human strains of *Enterobacteriaceae* from different parts of the world, high-level sulphonamide resistance in all of the isolates studied was accounted for by presence of sul1, sul2 or both.2
The new resistant dihydropteroate synthase, Sul3, shares an amino acid identity of ∼40% with previously known resistant enzymes. The sul3 gene was originally identified on a 54 kb conjugative plasmid, but it also appeared to be harboured by a larger plasmid different from the first. In our studies, sul3 was carried by a large plasmid in one of the two isolates. The location of the sul3 gene on this plasmid was shown by conjugation experiments where sul3 and sulphonamide resistance were transferred with the plasmid to the recipient strains. Whether the new sul3 gene originated in human or animal pathogens is important. It might not be a coincidence that sul3 was first identified in pig isolates since the consumption of sulphonamides for veterinary use is relatively widespread. The sul3 gene has also been reported recently in E. coli isolates from cattle, pigs and poultry in Germany. Among the human isolates tested in our study, however, sul3 still seemed to be rare.

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References