Effect of long-term trimethoprim/sulfamethoxazole treatment on resistance and integron prevalence in the intestinal flora: a randomized, double-blind, placebo-controlled trial in children

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Objectives: The aim of this study was to test the hypothesis that trimethoprim/sulfamethoxazole selects for integron-positive and multidrug-resistant Enterobacteriaceae in the intestinal flora.

Methods: During 1 year of follow-up, antibiotic susceptibility and the presence of integrons were determined in faecal Enterobacteriaceae isolated from 99 children with chronic active otitis media, randomly assigned to treatment with trimethoprim/sulfamethoxazole or placebo (http://www.clinicaltrials.gov; trial registration number NCT00189098).

Results: At 6 and 12 weeks of follow-up, 32 (91%) and 24 (67%) children in the trimethoprim/sulfamethoxazole group carried trimethoprim/sulfamethoxazole-resistant Enterobacteriaceae versus 10 (21%) and 8 (17%) children in the placebo group [rate differences (RDs): 70 (95% CI: 55; 85) and 50 (95% CI: 31; 69), respectively. Multiresistance also increased during trimethoprim/sulfamethoxazole treatment. At 6 weeks of follow-up, the integron prevalence was 26 (79%) in the trimethoprim/sulfamethoxazole group and 10 (22%) in the placebo group [RD: 57 (95% CI: 39; 75)]. After 12 weeks the integron prevalence, and after 1 year the susceptibility levels, had returned to baseline values.

Conclusions: Initially, trimethoprim/sulfamethoxazole usage was strongly associated with the appearance of integron-positive (multi)drug-resistant Enterobacteriaceae in the intestinal flora. After prolonged exposure to trimethoprim/sulfamethoxazole, however, this population of Enterobacteriaceae was substituted by a population with non-integron-associated resistance mechanisms. After trimethoprim/sulfamethoxazole was discontinued, susceptibility rates to all antibiotics returned to baseline levels.

Keywords: drug resistance, Enterobacteriaceae, intestine

Introduction

The intestinal tract is an important reservoir of many bacterial pathogens allowing transfer of antimicrobial resistance genes within and across bacterial species. Selective pressure exerted by antimicrobial usage is considered crucial to the emergence and dissemination of antibiotic-resistant strains in the intestinal tract.

Trimethoprim/sulfamethoxazole is widely used for long-term prophylaxis and treatment of infections of the urinary and respiratory tracts. This widespread use is associated with increasing resistance rates to trimethoprim/sulfamethoxazole.

Most trimethoprim/sulfamethoxazole resistance genes in Enterobacteriaceae reside within class 1 integrons, genetic elements that frequently reside in horizontally transferable elements and play an important role in the dissemination of resistance genes. Integrons are capable of recognizing, capturing and expressing multiple resistance genes in cassette structures and are strongly associated with multidrug resistance. Class 1 integrons are the most common integrons among Enterobacteriaceae and usually contain a sul1 gene encoding resistance to sulfamethoxazole, alongside the mobile gene cassettes. The aim of this study was to test the hypothesis that by oral use of trimethoprim/sulfamethoxazole...
sulfamethoxazole, the sulfamethoxazole component will select for class 1 integron-positive strains, leading to the presence of clinically relevant concentrations of integron-positive multidrug-resistant Enterobacteriaceae in the intestinal flora.

Patients and methods

Patients

From February 2003 until June 2006, faecal samples were collected from 99 children with chronic active otitis media who participated in a randomized placebo-controlled trial on the effectiveness of sulfamethoxazole/trimethoprim (http://www.clinicaltrials.gov/; trial registration number NCT00189098). Inclusion criteria were age of 1–12 years and a documented history of more than 3 months of continuous otorrhoea through either a tympanic membrane perforation or a tympanostomy tube. Children with cholesteatoma, known immune deficiency other than for IgA or IgG subclasses, Down’s syndrome, craniofacial anomalies, cystic fibrosis, primary ciliary dyskinesia, allergy to trimethoprim/sulfamethoxazole or continuous use of antibiotics for 6 weeks in the past 6 months were excluded from the study. Children whose parents gave informed consent (including to this substudy) were randomly assigned to either trimethoprim/sulfamethoxazole to be given orally (18 mg/kg, twice daily) or a placebo for 6 weeks. The investigators remained blinded to the randomization until the end of the study. If otorrhoea was present in either ear at the first follow-up visit at 6 weeks, the study medication was continued for a further 6 weeks. The study medication was discontinued at this first follow-up visit if both ears were free from otorrhoea. Parents were instructed to restart the study medication if symptoms of otorrhoea recurred between the follow-up visits at 6 and 12 weeks. At the 12 week follow-up visit, all study medication was discontinued.

Further details regarding the randomization procedure, allocation concealment and the results concerning the primary outcome, i.e. otorrhoeal symptoms, are described elsewhere. To study the effect of trimethoprim/sulfamethoxazole on the proportion of children with integron-positive and (multi)drug-resistant Enterobacteriaceae in their intestinal tract (secondary outcome), faecal samples were collected at study entry and 6 and 12 weeks and 1 year of follow-up. To determine whether susceptibility patterns of the isolates from the trial participants differed from those of children in the open population, we also collected faecal samples from 57 healthy children attending a daycare centre or primary school in the vicinity of Utrecht, The Netherlands. The medical Ethics Committee of the University Medical Center Utrecht approved the study protocol, including this substudy.

Microbiological investigation

Since resistance to sulfamethoxazole is a very sensitive screening criterion for the detection of integrons in Enterobacteriaceae, faecal samples from patients and controls were cultured for Gram-negative bacteria on MacConkey agar with sulfamethoxazole (512 mg/L). As a control for the adequacy of the culture conditions, all samples were cultured on MacConkey agar without sulfamethoxazole as well. From each plate, all morphologically different colonies were subcultured for further investigation with a minimum of three colonies per plate. Identification and susceptibility were tested using the Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD, USA). Susceptibility to the following antimicrobials was determined: trimethoprim/sulfamethoxazole, ciprofloxacin, trimethoprim, gentamicin, amoxicillin, amoxicillin/clavulanate, cefuroxime, ceftriaxone, nitrofurantoin, colistin, chloramphenicol and meropenem. Extended-spectrum β-lactamase (ESBL) production was determined for *Escherichia coli* and *Klebsiella* spp. using Etest (AB Biodisk, Solna, Sweden) ESBL. Additional testing for susceptibility to sulfamethoxazole was performed using the agar disc diffusion method. Breakpoints were those recommended by the CLSI. All not fully susceptible isolates were grouped together with the resistant isolates. Multiresistance was defined as resistance to at least two antimicrobial classes.

All isolated Enterobacteriaceae resistant to sulfamethoxazole were tested for the presence of class 1 integrons by PCR amplification of the class 1 integrase-specific *intI* gene (GenBank accession no. M73819), as described previously. The primers were *IntI*-F (5’,-TTCGAGTTAACATCAAGG-3’) and *IntI*-R (5’,-AGGAGATCCGAAGACCTC-3’).

Statistical analysis

The power of the study was calculated according to the primary outcome, i.e. otorrhoeal signs of otitis media at 12 weeks of follow-up. Assuming a spontaneous recovery of otorrhoea of 25%, a treatment effect of trimethoprim/sulfamethoxazole of 50% (based on a retrospective study of children treated with trimethoprim/ sulfamethoxazole for chronic active otitis media at our hospital) and using an α of 0.05 and a power of 0.80, it was calculated that each group should consist of 50 children. Rate differences with 95% confidence intervals were calculated at baseline and at the three follow-up visits to compare the children with chronic otitis media and the healthy control group for the prevalences of Enterobacteriaceae, antibiotic resistance and integrons. Duration of treatment with trimethoprim/sulfamethoxazole varied as the study medication was continued at 6 weeks of follow-up if otorrhoea was present and discontinued if the ear was dry. Therefore, subanalyses were performed for the results at 12 and 52 weeks of follow-up for

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Trimethoprim/sulfamethoxazole (n=48)</th>
<th>Placebo (n=51)</th>
<th>Healthy controls (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>28 (58)</td>
<td>27 (53)</td>
<td>28 (51)</td>
</tr>
<tr>
<td>Mean age in years (SD)</td>
<td>4.5 (3.4)</td>
<td>3.9 (2.8)</td>
<td>3.6 (2.3)</td>
</tr>
<tr>
<td>Daycare or school attendance in year before study entry</td>
<td>43 (90)</td>
<td>47 (92)</td>
<td>55 (100)</td>
</tr>
<tr>
<td>Use of systemic antibiotics in year before study entry</td>
<td>46 (96)</td>
<td>46 (90)</td>
<td>20 (36)</td>
</tr>
<tr>
<td>Use of systemic antibiotics in the last 2 weeks before study entry</td>
<td>2 (4)</td>
<td>2 (4)</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of patients with chronic active otitis media randomized to trimethoprim/sulfamethoxazole or placebo and healthy control children; number (%)
those who had received trimethoprim/sulfamethoxazole from study entry until the follow-up visit at 6 weeks (SXT-I) and for those who continued or restarted trimethoprim/sulfamethoxazole between 6 and 12 weeks of follow-up (SXT-II).

Results

Baseline characteristics of the trial participants and the healthy controls are summarized in Table 1. As expected, fewer children in the healthy control group had used systemic antibiotics in the previous year. Figure 1 shows the flow chart of the number of eligible patients and those that consented to participate, as well as the numbers of participants with complete follow-up and the dropouts. Of the 48 children in the trimethoprim/sulfamethoxazole group, 20 (42%) discontinued (SXT-I) and 28 (58%) children continued (SXT-II) the study medication between 6 and 12 weeks of follow-up.

Table 2 shows the number (%) of children with faecal samples from which Enterobacteriaceae were isolated in the trimethoprim/sulfamethoxazole, the placebo and the healthy control groups with positive cultures for Enterobacteriaceae at baseline and at the

Figure 1. Flow of participants through the substudy of the trial. *The number exceeds 90 because more than one reason could be indicated. SXT-I, children with chronic active otitis media treated with trimethoprim/sulfamethoxazole from study entry up to the follow-up visit at 6 weeks; SXT-II, children with chronic active otitis media who were also treated with trimethoprim/sulfamethoxazole between 6 and 12 weeks of follow-up; T6, 6 weeks of follow-up; T12, 12 weeks of follow-up.
follow-up visits. The high detection rates at baseline and during follow-up in the placebo group reflect the adequacy of the culture conditions. The most frequently cultured species during the study were *E. coli* (90% of faecal samples), followed by *Klebsiella* spp. (8%). Non-fermenting Gram-negative bacteria were cultured from <10% of the faecal samples. The distribution of species did not change during the study and did not differ between the trimethoprim/sulfamethoxazole group and the placebo group.

During the study, antimicrobials involved in multidrug resistance were mainly sulfamethoxazole, trimethoprim/sulfamethoxazole, amoxicillin and amoxicillin/clavulanic acid. Resistance to aminoglycosides and ciprofloxacin was found sporadically. No resistance was observed to meropenem and no ESBL-positive strains were identified.

At baseline, antibiotic resistance and multiresistance rates were similar for the trimethoprim/sulfamethoxazole group, the placebo group and the healthy control group.

At 6 weeks of follow-up, 32 (91%) Enterobacteriaceae-positive cultures were resistant to trimethoprim/sulfamethoxazole in the trimethoprim/sulfamethoxazole group versus 10 (21%) in the placebo group (Figure 2). Multidrug resistance was present in 34 (97%) Enterobacteriaceae-positive cultures in the trimethoprim/sulfamethoxazole group versus 23 (49%) in the placebo group (Figure 3).

At 12 weeks of follow-up, 24 (67%) Enterobacteriaceae-positive cultures were resistant to trimethoprim/sulfamethoxazole in the trimethoprim/sulfamethoxazole group versus 8 (17%) in the placebo group (Figure 2). Multidrug resistance was present in 26 (72%) Enterobacteriaceae-positive cultures in the trimethoprim/sulfamethoxazole group versus 26 (55%) in the placebo group (Figure 3). Within the trimethoprim/sulfamethoxazole group, the children who continued trimethoprim/sulfamethoxazole therapy between 6 and 12 weeks of follow-up (SXT-II) had higher resistance rates: 19 (86%) Enterobacteriaceae-positive cultures were resistant to trimethoprim/sulfamethoxazole in the SXT-II group versus 5 (36%) in the SXT-I group. Multidrug resistance was found in 26 (91%) Enterobacteriaceae-positive cultures of the SXT-II group and in 6 (43%) of the SXT-I group.

At 1 year of follow-up, the proportion of Enterobacteriaceae-positive cultures resistant to trimethoprim/sulfamethoxazole as well as multidrug resistance returned to baseline levels.

The susceptibility patterns for sulfamethoxazole and amoxicillin followed the same pattern as that for trimethoprim/sulfamethoxazole.

### Table 2. Number (%) of children with faecal samples from which Enterobacteriaceae were isolated

<table>
<thead>
<tr>
<th></th>
<th>Inclusion</th>
<th>6 weeks</th>
<th>12 weeks</th>
<th>1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>53 (96)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Placebo group</td>
<td>47 (92)</td>
<td>47 (100)*</td>
<td>47 (98)*</td>
<td>34 (85)</td>
</tr>
<tr>
<td>Trimethoprim/sulfamethoxazole group</td>
<td>47 (98)</td>
<td>35 (74)*</td>
<td>36 (84)*</td>
<td>36 (97)</td>
</tr>
<tr>
<td>SXT-I</td>
<td></td>
<td>14 (82)</td>
<td>15 (94)</td>
<td></td>
</tr>
<tr>
<td>SXT-II</td>
<td></td>
<td>22 (85)</td>
<td>21 (100)</td>
<td></td>
</tr>
</tbody>
</table>

*Rate difference: −26 (95% CI: −39; −13).

Rate difference: −14 (95% CI: −26; −2).

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At 1 year of follow-up, integron prevalences returned to baseline in all groups.

Figure 2. Proportion of children with trimethoprim/sulfamethoxazole-resistant Enterobacteriaceae at inclusion and during follow-up. *P < 0.05; #P < 0.05; ^P < 0.05. SXT total, children with chronic active otitis media treated with trimethoprim/sulfamethoxazole for 6–12 weeks (SXT-I + SXT-II); SXT-I, children with chronic active otitis media treated with trimethoprim/sulfamethoxazole from study entry up to the follow-up visit at 6 weeks; SXT-II, children with chronic active otitis media who were also treated with trimethoprim/sulfamethoxazole between 6 and 12 weeks of follow-up; T6, 6 weeks of follow-up; T12, 12 weeks of follow-up; T1 year, 1 year of follow-up.

At baseline, the proportion of children with integron-positive Enterobacteriaceae was similar in the trimethoprim/sulfamethoxazole group, the placebo group and the healthy control group (Figure 4). At 6 weeks of follow-up, more children in the trimethoprim/sulfamethoxazole group than in the placebo group carried integron-positive Enterobacteriaceae: 26 (79%) and 10 (22%), respectively. At 12 weeks of follow-up, these percentages decreased to 32% in the trimethoprim/sulfamethoxazole group and 11% in the placebo group. Subanalysis of the data at 12 weeks for the SXT-I and SXT-II groups did not alter the results.

At 1 year of follow-up, integron prevalences returned to baseline in all groups.

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**Figure 3.** Proportion of children with multiresistant Enterobacteriaceae at inclusion and during follow-up. *P*<0.05; #P<0.05. SXT total, children with chronic active otitis media treated with trimethoprim/sulfamethoxazole for 6–12 weeks (SXT-I + SXT-II); SXT-I, children with chronic active otitis media treated with trimethoprim/sulfamethoxazole from study entry up to the follow-up visit at 6 weeks; SXT-II, children with chronic active otitis media who were also treated with trimethoprim/sulfamethoxazole between 6 and 12 weeks of follow-up; T6, 6 weeks of follow-up; T12, 12 weeks of follow-up; T1 year, 1 year of follow-up.

**Figure 4.** Proportion of children with integron-positive Enterobacteriaceae at inclusion and during follow-up. *P*<0.05. SXT total, children with chronic active otitis media treated with trimethoprim/sulfamethoxazole for 6–12 weeks (SXT-I + SXT-II); SXT-I, children with chronic active otitis media treated with trimethoprim/sulfamethoxazole from study entry up to the follow-up visit at 6 weeks; SXT-II, children with chronic active otitis media who were also treated with trimethoprim/sulfamethoxazole between 6 and 12 weeks of follow-up; T6, 6 weeks of follow-up; T12, 12 weeks of follow-up; T1 year, 1 year of follow-up.

**Discussion**

To our knowledge, this is the first study that evaluates the effects of trimethoprim/sulfamethoxazole on the intestinal flora alongside a randomized placebo-controlled trial and the first study to report the effect of prolonged oral use of trimethoprim/sulfamethoxazole on the prevalence of integrons in Enterobacteriaceae in the intestinal flora.

So far, only a few prospective studies have investigated the effect of trimethoprim/sulfamethoxazole on Enterobacteriaceae in the intestinal tract. A possible explanation for the less pronounced effect in our study was the relatively high proportion of carriage of trimethoprim/sulfamethoxazole-resistant strains among children at inclusion (around 20%).

The use of trimethoprim/sulfamethoxazole caused a temporary increase in the proportion of children with (multi-)resistant Enterobacteriaceae, a finding in accordance with previous observations. Since the intestinal tract is an important reservoir of many bacterial pathogens, it can be assumed that these children are more prone to acquire an infection with (multi-)resistant Enterobacteriaceae. The recent identification of antimicrobial prophylaxis as a risk factor for antimicrobial resistance among children with recurrent urinary tract infections supports this assumption.

The increased (multi)drug resistance rates at 6 weeks of follow-up were associated with an increased prevalence of integrons, supporting the hypothesis that by oral administration of trimethoprim/sulfamethoxazole, the sulfamethoxazole component selects for class 1 integron-positive multidrug-resistant Enterobacteriaceae in the intestinal flora. This association was also found between trimethoprim/sulfamethoxazole treatment and the occurrence of _sul_ genes and increased resistance to trimethoprim/sulfamethoxazole in _E. coli_ in urines from children with recurrent urinary tract infections.

Surprisingly, at 12 weeks of follow-up, the proportion of children with integron-positive strains had returned to baseline levels despite continued trimethoprim/sulfamethoxazole therapy and while the strains retained high resistance levels. Apparently, between 6 and 12 weeks of follow-up, integron-negative Enterobacteriaceae substituted the integron-positive Enterobacteriaceae with non-integron-associated resistance mechanisms. This finding can be explained by the concept that low antibiotic concentrations may produce a substantial stress in the bacterial population leading to increased mutation rates and transfer of resistance genes stepwise, resulting in more diverse and effective adaptive responses.

After trimethoprim/sulfamethoxazole was discontinued, resistance rates to all antibiotics returned to baseline levels consistent with the findings of the one study that also followed their participants after discontinuation of therapy. Further research is necessary to establish whether the patients really lost their resistant strains or whether the resistant strains only decreased below detection levels to possibly re-emerge following a further antimicrobial course.
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A possible limitation of this study is the limited sensitivity of the detection method of the integron-positive Enterobacteriaceae used in this study; more colonies per plate or higher inocula would have increased the sensitivity of the detection method. However, we think that the detection method used was adequate. The aim of this study was to determine the change in the proportion of children with integron-positive multidrug-resistant strains, and using this method a significant increase was found.

Conclusions

Trimethoprim/sulfamethoxazole usage was initially strongly associated with the appearance of integron-positive (multi)drug-resistant Enterobacteriaceae in the intestinal flora. After prolonged exposure to trimethoprim/sulfamethoxazole, however, this population of Enterobacteriaceae was substituted by a population with non-integron-associated resistance mechanisms. The presence of resistant Enterobacteriaceae in the intestinal tract during therapy most likely results in a higher risk of acquisition of infections caused by these (multi)resistant strains. Further studies are needed to determine both the presence and the duration of such increased risk after discontinuation of therapy.

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Transparency declarations

None of the authors has any conflict of interest.


References