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Within-Population Distribution of Trimethoprim Resistance in *Escherichia coli* before and after a Community-Wide Intervention on Trimethoprim Use

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A 2-year prospective intervention on the prescription of trimethoprim reduced the use by 85% in a health care region with 178,000 inhabitants. Here, we performed before-and-after analyses of the within-population distribution of trimethoprim resistance in *Escherichia coli*. Phylogenetic and population genetic methods were applied to multilocus sequence typing data of 548 consecutively collected *E. coli* isolates from clinical urinary specimens. Results were analyzed in relation to antibiotic susceptibility and the presence and genomic location of different trimethoprim resistance gene classes. A total of 163 *E. coli* sequence types (STs) were identified, of which 68 were previously undescribed. The isolates fell into one of three distinct genetic clusters designated BAPS 1 (*E. coli* phylogroup B2), BAPS 2 (phylogroup A and B1), and BAPS 3 (phylogroup D), each with a similar frequency before and after the intervention. BAPS 2 and BAPS 3 were positively and BAPS 1 was negatively associated with trimethoprim resistance (odds ratios of 1.97, 3.17, and 0.26, respectively). In before-and-after analyses, trimethoprim resistance frequency increased in BAPS 1 and decreased in BAPS 2. Resistance to antibiotics other than trimethoprim increased in BAPS 2. Analysis of the genomic location of different trimethoprim resistance genes in isolates of ST69, ST58, and ST73 identified multiple independent acquisition events in isolates of the same ST. The results show that despite a stable overall resistance frequency in *E. coli* before and after the intervention, marked within-population changes occurred. A decrease of resistance in one major genetic cluster was masked by a reciprocal increase in another major cluster.

During the past 70 years, the use and misuse of antibiotics have prompted bacterial adaptation through mutations and acquisition of foreign DNA, leading to antibiotic resistance. Increasing levels of bacterial antibiotic resistance now is an increasing cause of morbidity and mortality in humans (1, 2). The acquisition of plasmids with resistance genes has resulted in a dramatic, almost explosive increase of resistance among *Enterobacteriaceae* and *Escherichia coli* in particular (1, 3).

E. coli is a facultative pathogen residing in the intestines of birds and mammals (4) with the potential to cause urinary tract infections (UTIs), gastroenteritis, and septicemia in humans (5, 6). The urinary tract is the most frequently infected extraintestinal site.

Nucleotide sequence-based bacterial typing methods, like multilocus sequence typing (MLST) and whole-genome sequencing, have shown the evolution of *E. coli* to be shaped by clonal inheritance and lateral gene transfer (7–9). Laboratory investigations combined with mathematical modeling have provided knowledge on the lateral gene transfer of resistance between well-defined bacterial strains and on the responses of experimentally defined bacterial populations to antibiotic exposure (10, 11). There is, however, little information on how bacterial populations in the world at large change over time with change of antibiotic exposure. Several reports have addressed the relation between antibiotic use at the national level and the corresponding levels of antibiotic resistance in the community. In some cases, there was support for a correlation, but most often substantial amounts of unexplained variations suggest that there are many additional factors involved (12–14). Previous studies addressing changes in the *E. coli* population have focused on the epidemiology of specific resistant clones and their spread. Six *E. coli* sequence types (STs) have been associated with specific resistance traits. ST69, also known as clonal group A (CGA), is associated with trimethoprim-sulfamethoxazole resistance (15), and ST393 (O15:K52:H1) is associated with multiple antibiotic resistance, including trimethoprim resistance (16). Resistance to third-generation cephalosporins, fluoroquinolones, and/or carbapenems has been associated with ST131 (17–19), ST38 (20–24), ST405 (17, 25, 26), and ST648 (22, 26, 27).

Here, we have studied a large prospective 2-year antibiotic intervention with an overall reduction in the use of trimethoprim (including trimethoprim-sulfamethoxazole) in the Kronoberg

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health care region of Sweden, including 25 primary health care centers and two hospitals, from 2004 to 2006. The use of trimethoprim dropped 85% during the intervention, while the use of pivmecillinam, nitrofurantoin, and ciprofloxacin increased. The level of trimethoprim resistance in *E. coli* and the overall distribution of *dfr* genes encoding trimethoprim resistance remained stable (28, 29). Coselection from concomitant resistance to ampicillin and ciprofloxacin, both frequently used antibiotics during the intervention, and a low fitness cost of trimethoprim resistance were put forward to explain that the trimethoprim resistance rate did not decrease during the intervention. To investigate the genetic structure of the *E. coli* population before and after the intervention, we here performed an MLST-based study on trimethoprim-susceptible and trimethoprim-resistant *E. coli* isolates collected before and after the intervention to answer the following questions: (i) Were there changes in the genetic diversity between the start point and endpoint of the intervention? (ii) Could the persisting levels of trimethoprim resistance all through the intervention period be explained by the presence of specific trimethoprim-resistant and evolutionary successful clones? (iii) Were trimethoprim resistance genes equally distributed between different *E. coli* subpopulations?

MATERIALS AND METHODS

Patients and isolates. All isolates were collected during the trimethoprim intervention study performed in the Kronoberg health care region, Sweden, from 1 October 2004 to 30 September 2006 (29). All *Enterobacteriaceae* isolated from urinary specimens from June 2004 through December 2008 ($n = 27{,}777$) were stored frozen. From this collection, 100 consecutive trimethoprim-resistant and 174 consecutive trimethoprim-susceptible *E. coli* isolates were identified in two time periods: June to September 2004 (before the intervention) and June to September 2006 (after the intervention). Only one *E. coli* isolate per patient and time period was included. None of the patients contributed an isolate to both time periods. Information regarding, age, sex, origin of the specimen (i.e., community or hospital), and specimen type (voided urine or catheter urine) was available for all isolates ($n = 548$). Clinical information as reported at referral (i.e., asymptomatic bacteriuria, cystitis, or pyelonephritis) was available for 63% ($n = 346$) of the isolates. The study was approved by the regional ethics committee of Linköping University, Sweden (approval no. 03-04).

Identification of *E. coli* and susceptibility testing. Species identification and antibiotic susceptibility testing with ampicillin, amdinocillin (mecillinam), trimethoprim, cefadroxil, nitrofurantoin, and nalidixic acid were performed as previously described (29).

DNA preparation, generation of MLST data, and detection of trimethoprim resistance genes. *E. coli* was grown on horse blood agar plates overnight, and DNA was isolated using an automated preparation system (Abbot m2000sp; Abbot Park, IL, USA). The MLST scheme described by Wirth et al. (9), available at http://mlst.warwick.ac.uk, was used for the detection of seven gene fragments of housekeeping genes. PCRs were performed in a TGradient thermocycler (Biometra GmbH, Gottingen, Germany). For sequencing, the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was used before analysis in the 3730xl DNA analyzer (Applied Biosystems). All sequence traces were imported, aligned, trimmed, and quality control aided by functions of the software BioNumerics v.6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium). MLST allele designations were determined via the electronic MLST database. Novel ST designations were provided by the curator of the database. The trimethoprim-resistant isolates ($n = 200$) had been analyzed for the presence of 13 *dfr* genes encoding resistance to trimethoprim as previously described (28).

Analysis of nucleotide sequence data. The nucleotide sequences of seven gene fragments were aligned and concatenated for inferring phylogenetic relationships. The software MEGA4 was used for constructing a neighbor-joining (NJ) tree using default settings with the maximum composite likelihood model, assuming rate uniformity and pattern homogeneity to estimate evolutionary distances between sequences (30). For identification of genetically isolated clusters within the *E. coli* population that may have specific characteristics with respect to antibiotic resistance, the software BAPS 5.3 using a Bayesian clustering method was applied using the "clustering with linked loci" option and the MLST type of "indata" (31). The software is designed to estimate the number of subpopulations (genetic clusters) represented in the data and to evaluate the extent of recombination between clusters. Initially, the software was run with the maximum number of subpopulations set at a K value of 10. The log marginal likelihood value with respect to K was then estimated using five runs for each fixed K value between 2 and 8. Finally, minimal spanning trees (MST) were constructed in BioNumerics v.6.0 (Applied Maths NV) using the option for categorical data with single- and double-locus variant priority rules.

Presence and plasmid location of *dfrA1*, *dfrA5*, *dfrA17*, and bla_{TEM-1}. Isolates with a previously verified presence of *dfrA1* ($n = 5$), *dfrA5* ($n = 5$), and *dfrA17* ($n = 5$) (28) were analyzed for the presence of bla_{TEM-1} genes using specific PCR as previously described (32). The genetic location of resistance was determined by S1 nuclease digestion of total DNA and pulsed-field gel electrophoresis (33). In brief, plasmid sizes were determined on agarose gels and blotted onto positively charged nylon membranes, and the locations of antibiotic resistance genes were detected by PCR-generated probes for *dfrA1*, *dfrA5*, *dfrA17*, and bla_{TEM-1}.

Identification of uropathogenic lineages in *E. coli*: CGA and O15:K52:H1. Using single-nucleotide polymorphisms of the *fumC* sequences obtained in the MLST, all isolates were screened for the presence of C288T (specific to CGA) and G594A (specific to O15:K52:H1) (34, 35).

Statistical analysis. Fisher's exact test and the chi-square test were used to compare the proportion of resistance traits between different genetic subpopulations of *E. coli*, i.e., the different STs and BAPS clusters defined among the 548 isolates. The same tests were used to analyze associations of STs containing ≥ 10 isolates with patient age groups (0 to 12, 13 to 29, 30 to 49, 50 to 69, 70 to 89, or >90 years old), gender, hospital or community origin of infection, specimen type (urinary catheter or voided), and infection type (asymptomatic bacteriuria, cystitis, or pyelonephritis). Throughout the analyses, no mathematical correction was made for multiple comparisons. For investigating change in resistance over time, a test strategy was used to take into account that fixed numbers of trimethoprim-resistant ($n = 100$) and -susceptible ($n = 174$) bacterial isolates were collected in 2004 and in 2006. Odds ratios (OR; i.e., the fraction of resistant isolates in year 2006 divided by the fraction of resistant isolates in year 2004) were calculated and tested under the null hypothesis of no change between the samplings. A parametric bootstrap test was used for estimating the random distribution of odds ratios. Simulated bootstrap sampling of two binomial distributions with a common parameter estimating the frequency of resistance (i.e., the fraction of resistant isolates in the combined data set) was performed. The sampling was iterated for 10,000 generations with calculation of the odds ratio each time. The Simpson index of diversity (1-D) was calculated for the *E. coli* population as a measure of genetic richness and evenness, using the formula $1 - [\Sigma n(n-1)/N(N-1)]$, where n is the number of isolates sharing an ST and N is the total number of isolates.

RESULTS

Descriptive epidemiology. During the intervention period, the annual rate of sampling for urine culture was 10,300 per 100,000 county residents. The proportions of trimethoprim-resistant *E. coli* isolates cultured from urinary specimens were similar just before the intervention (sampling period from 1 April to 30 September 2004; $n = 2{,}285$) and at the end of the intervention (1 April to 30 September 2006; $n = 2{,}184$). With frequencies of 0.1080 and

TABLE 1 Number of *E. coli* isolates and the odds ratios, measuring strength of association between genetic population and trimethoprim resistance[a]

Genetic population	No. of isolates		Odds ratio[b]	95% CI	Median age of patients in yrs (range)	No. of females/ males (ratio)
	Trimethoprim resistant	Trimethoprim susceptible				
BAPS 1	81	250	**0.26**	0.18–0.38	62 (0–96)	281/50 (5.6)
BAPS 2	64	57	**1.97**	1.30–3.05	71 (0–98)	114/7 (16.3)
BAPS 3	55	39	**3.17**	1.96–5.17	64 (0–97)	83/11 (7.5)
ST12 (BAPS 1)	4	16	0.42	0.10–1.34	58 (0–96)	13/7
ST14 (BAPS 1)	2	5	0.69	0.065–4.28	61 (6–81)	7/0
ST73 (BAPS 1)	24	65	0.59	0.34–1.00	64 (0–94)	74/15
ST80 (BAPS 1)	4	13	0.53	0.12–1.74	62 (4–78)	16/1
ST95 (BAPS 1)	4	21	**0.32**	0.08–0.96	68 (1–94)	25/0
ST127 (BAPS 1)	6	26	**0.38**	0.13–0.98	60 (18–92)	29/3
ST131 (BAPS 1)	12	7	**3.10**	1.10–9.47	64 (5–90)	18/1
ST141 (BAPS 1)	3	28	**0.17**	0.03–0.58	66 (1–94)	22/9
ST144 (BAPS 1)	0	6	Approaches 0		62 (6–82)	6/0
ST10 (BAPS 2)	16	9	**3.27**	3.33–8.57	64 (1–98)	25/1
ST58 (BAPS 2)	9	1	**16.3**	2.23–718.7	60 (18–96)	10/0
ST88 (BAPS 2)	6	6	1.76	0.46–6.69	67 (29–82)	12/0
ST540 (BAPS 2)	2	3	1.16	0.096–1.23	79 (66–94)	3/2
ST38 (BAPS 3)	3	4	1.31	0.19–7.82	69 (22–90)	5/2
ST59 (BAPS 3)	2	7	0.49	0.05–2.62	67 (3–85)	8/1
ST69 (BAPS 3)	26	9	**5.61**	2.48–13.92	53 (0–94)	33/2
ST393 (BAPS 3)	5	0	Plus infinity		56 (19–92)	4/1
ST405 (BAPS 3)	6	1	10.7	1.28–495	77 (28–80)	7/0
Other STs	66	121	0.92	0.63–1.34	69 (0–97)	165/23
Total	200	348			64 (0–98)	482/68 (7.09)

[a] Corresponding patients' ages and gender distributions are shown.
[b] Bold text signifies a statistically significant effect size.

0.1181 for the respective period, the 2-sample chi-square test for equality of proportions indicated no change ($P = 0.3085$).

A total of 548 *E. coli* isolates were genetically and phenotypically characterized, originating from the same number of patients with a mean and median age of 64 years (range of 0 to 98) and a female-to-male ratio of 7.07 (Table 1). Before the intervention, 16% of the samples originated from institutions located in the hospital, while 84% were from the community. The distribution was similar after the intervention, 14% and 86%, respectively. Age groups, gender, urinary catheter prevalence, and the type of infection were also similar (data not shown).

Genetic structure of the *E. coli* population. A total of 163 STs were identified among the 548 isolates; 68 of these were not previously characterized. Eighteen STs were represented by five or more isolates (Table 1), while 116 STs were represented by single isolates. Several STs previously reported to be commonly found in urinary tract infection (UTI) were represented by multiple isolates in our material (e.g., ST10, ST12, ST69, ST73, ST95, ST131, and ST393). Based on the sequences of the seven gene fragments of each isolate, an NJ tree was calculated to display genetic relationships. There was a broad genetic diversity among the isolates with three distinctive ST clusters, with two STs, ST1338 and ST1422, diverging strongly from all other STs (Fig. 1). The diverging ST1338 and ST1422, representing single isolates from 2004 and 2006, respectively, were susceptible to all tested antibiotics and subsequently identified as *Escherichia albertii* and *E. coli* cryptic clade III by analysis of the nucleotide sequences (D. Gordon, personal communication). Further population analysis by the BAPS software, which unlike NJ analysis also accounts for possible recombination events, defined a partition into different genetic subpopulations in agreement with the NJ analysis (Fig. 1). By repeated BAPS runs, we found that five genetic subpopulations provided the best fit to the data (three major subpopulations and two solitary genetic units). The estimated log marginal likelihood values for four, five, and six genetic subpopulations were −11,401.94, −11,360.67, and −11,498.82, respectively. A total of 161 STs were thus assigned to BAPS 1 ($n = 76$), BAPS 2 ($n = 57$), or BAPS 3 ($n = 28$), while both NJ and BAPS analysis put ST1338 and ST1422 as distinct and solitary genetic units. The rates of chromosomal sequence admixture within and between the three major BAPS subpopulations were estimated using the seven gene fragments. The in-between admixture was found to be low, reaching a maximum of 4% of housekeeping sequences with a net gene flow directed from BAPS 2 to BAPS 3 (Fig. 1). The within-BAPS cluster rates of admixture were 97, 99, and 94% for BAPS 1, BAPS 2, and BAPS 3, respectively.

The association of antibiotic resistance with genetic subpopulations. Trimethoprim resistance was unevenly distributed among the three BAPS clusters, and the odds ratios calculated here represent the strength of association between genetic subpopulation and trimethoprim resistance. There was a negative association for BAPS 1 (OR of 0.26) and positive association for BAPS 2 (OR of 1.97) and BAPS 3 (OR of 3.17) (Table 1). Increasing the genetic resolution, statistical analysis indicated ST69, ST10, ST131, ST58, ST405, and ST393 to contain greater proportions of trimethoprim-resistant isolates than those expected by chance (Table 1). Conversely, ST127, ST141, and ST95 were negatively associated with trimethoprim resistance. An analysis by gender showed

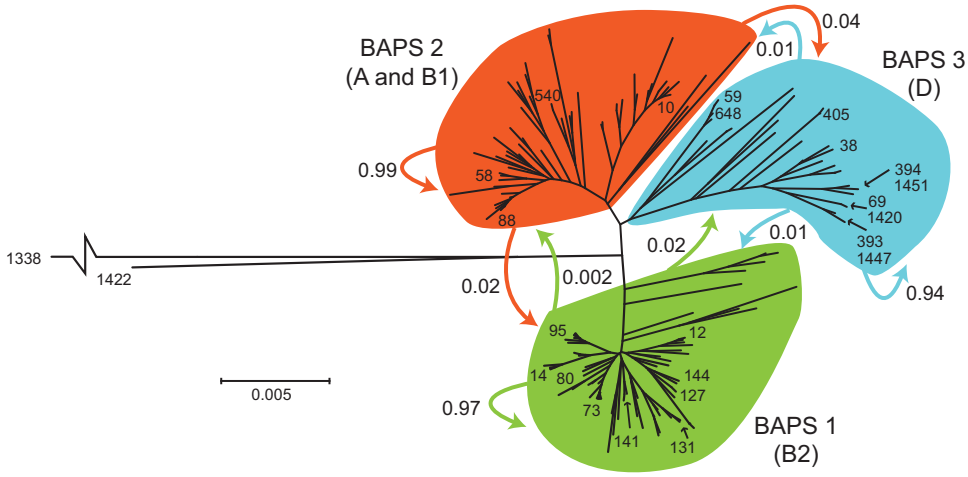

FIG 1 Genetic relationships displayed in an NJ tree with the 163 STs that were identified among 548 *E. coli* isolates. Selected STs have numerical labels at the tips of the tree. The three genetic populations identified by the software BAPS are shown by background coloring, with corresponding phylogroups in brackets. Rates of genetic admixture within and between BAPS populations are indicated by colored arrows. The scale bar shows the number of substitutions per nucleotide site. The branch of ST1338 is shortened due to space constraints.

a female patient predominance in all subpopulations, with particularly high female/male ratios for several STs of BAPS 2 (e.g., ST10, ST88, and ST58), and patients' ages were similar in different *E. coli* subpopulations (Table 1). There were similar proportions of isolates with a hospital origin in BAPS 1 (13%), BAPS 2 (17%), and BAPS 3 (17%). Out of the 200 trimethoprim-resistant isolates, 153 were ampicillin resistant, consistent with the frequent genetic linkage of *dfr* and bla_{TEM} genes.

Comparison of genetic structure before and after the intervention. Overall, the distribution of isolates into STs was highly similar between year 2004 and 2006, with assignment into 94 versus 106 different STs (Fig. 2). All the 28 STs represented by at least three isolates in the total material contained isolates from both 2004 and 2006. The Simpson index of genetic diversity was 0.949 (95% confidence interval [CI] of 0.935 to 0.963) among 274 isolates from 2004 and 0.958 (CI of 0.946 to 0.970) among 274 isolates from 2006. Separate analysis of trimethoprim-susceptible isolates showed a slightly lower diversity index at the beginning of the intervention (0.922 [95% CI of 0.898 to 0.947]) than at the end (0.959 [95% CI of 0.944 to 0.975]). The difference did not reach statistical significance. Similar levels of genetic diversity were calculated for the resistant isolates in 2004 and 2006, 0.954 (CI of 0.940 to 0.967) and 0.944 (0.930 to 0.958), respectively, indicating that there was no change in the number and relative abundance of different STs during the intervention.

The numbers of isolates assigned to each of the three main BAPS subpopulations were similar between the samplings, with 2004/2006 isolate number ratios of 1.08 ($P = 0.294$), 0.83 ($P = 0.303$), and 0.96 ($P = 0.91$) for BAPS 1, BAPS 2, and BAPS 3, respectively.

Shifts in antibiotic resistance frequencies among subpopulations. The proportion of isolates susceptible to all six tested antimicrobial agents decreased significantly between 2004 and 2006 among BAPS 1 isolates, while a concomitant increase was observed in BAPS 3 (Table 2). The odds ratio suggested an increase also in BAPS 2, although not statistically significant ($P = 0.132$). The proportion of trimethoprim-resistant isolates increased significantly in BAPS 1 and decreased in BAPS 2 (Table 2). For BAPS 3, the odds ratio suggested a decrease, although not statistically significant ($P = 0.089$). The proportion of antibiotic resistance to ampicillin, amdinocillin (mecillinam), cefadroxil, nitrofurantoin, and nalidixic acid among trimethoprim-sensitive isolates increased significantly in BAPS 2 but not in BAPS 1 or BAPS 3 (Table 2). At the genetic level of STs, the analysis of odds ratios for individual STs supported a shift in trimethoprim resistance from being common in STs of BAPS 2 and BAPS 3 but uncommon in BAPS 1 in year 2004 to be spread all over the three BAPS clusters in 2006. Of all analyzed STs, only ST80 of BAPS 1 showed a divergent pattern (Table 3).

The dispersal of trimethoprim resistance genes within respective subpopulation. A total of 191 trimethoprim-resistant isolates were previously tested positive for the 13 *dfr* genes tested: *dfrA1* ($n = 68$), *dfrA17* ($n = 44$), *dfrA5* ($n = 32$), *dfrA14* ($n = 16$), *dfrA12* ($n = 12$), *dfrA7* ($n = 8$), *dfrA8* ($n = 7$), *dfr2d* ($n = 1$), and *dfrA24* ($n = 1$) (28). Two isolates were positive for two different *dfr* genes, *dfrA1* and *dfrA5* for one and *dfrA1* and *dfrA17* for the other. The dispersal of different *dfr* genes among the STs of years 2004 and 2006 is presented in Fig. 2. The most common *dfr* genes were distributed widely throughout the *E. coli* population. Some associations to specific STs were observed. In the total material of 200 trimethoprim-resistant isolates, *dfrA17* was associated with ST69 ($P < 0.001$), *dfrA1* with ST73 ($P < 0.001$), and *dfrA5* with ST58 ($P = 0.002$). Analysis per *dfr* gene class and BAPS cluster disclosed that the 2004-to-2006 increase in the proportion of trimethoprim-resistant isolates in BAPS 1 and the reciprocal decrease in BAPS 2 (Table 2) were explained largely by the changes in prevalence of *dfrA1* (Table 4). There were fairly stable numbers of all other *dfr* gene classes belonging to different BAPS clusters between 2004 and 2006.

Plasmid or chromosomal location of trimethoprim and ampicillin resistance genes. The location of trimethoprim and ampicillin resistance genes on chromosome or plasmid was investigated in a subset of ST69, ST73, and ST58 isolates to address the possible clonal inheritance of resistance in these STs. We found that the genetic organizations of the *dfr* genes were very different among isolates of the same ST despite the association to the same

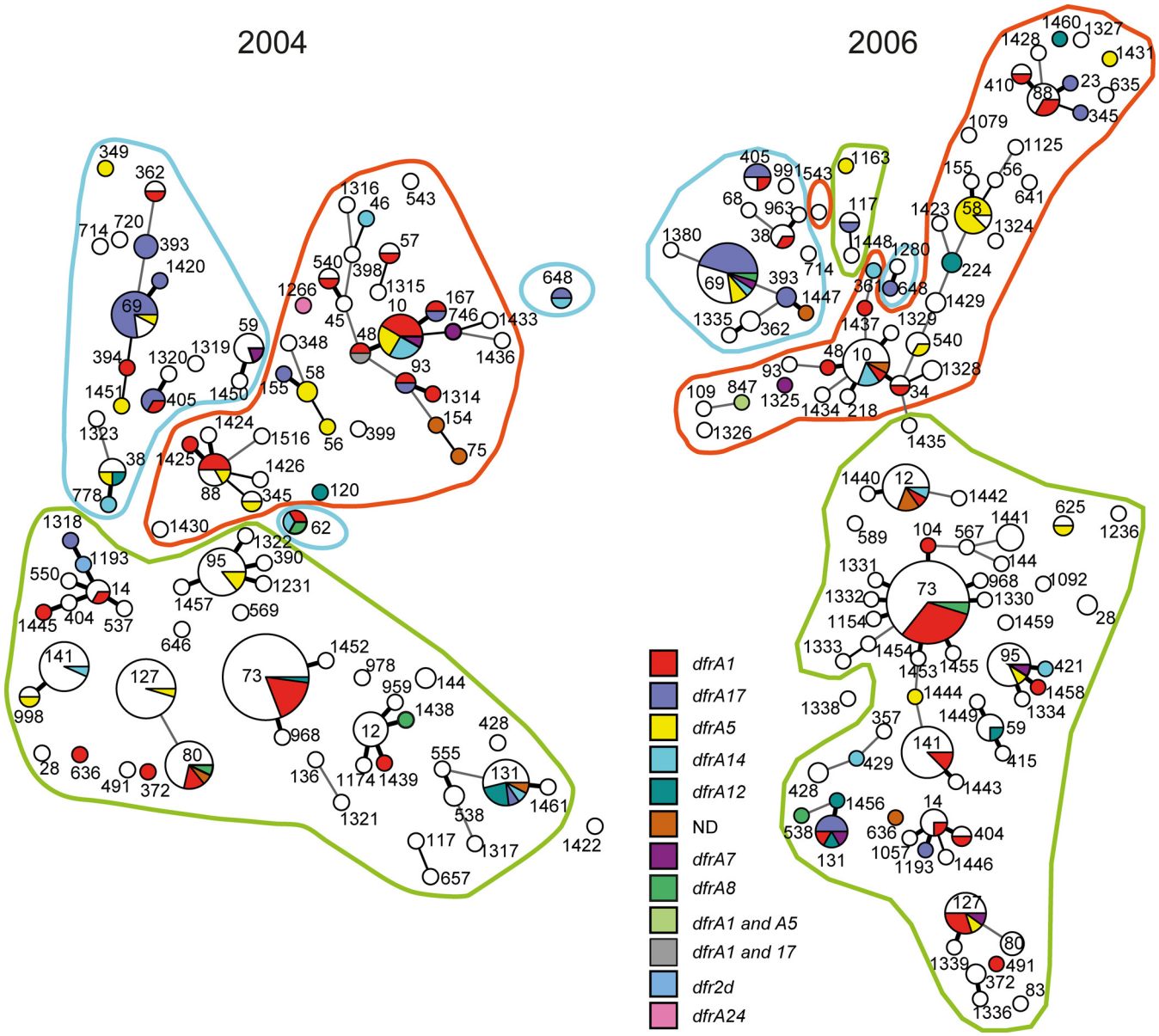

FIG 2 Minimum spanning trees representing the *E. coli* population before and after the intervention. The STs are labeled with numbers and represented as discs with sizes proportional to the number of isolates within them. The presence of different *dfr* gene classes is shown as colorized disc sectors. A line connecting a pair of STs means that nucleotide sequencing showed that they were identical at six (thick black lines), five (thin black lines), or four (thin gray lines) out of the seven gene fragments. Connecting lines representing less similarity were removed. The BAPS populations are enfolded by thick lines: BAPS 1, green; BAPS 2, orange; and BAPS 3, blue.

dfr gene class (Fig. 3). All isolates positive for *dfrA5* (ST58) and *dfrA17* (ST69) had a plasmid localization of the *dfr* gene, while the *dfrA1* genes in two out of six ST73 isolates analyzed were located on the chromosome. The number of plasmids per isolate ranged from 1 to 5 (ST69, 2 to 5; ST58, 1 or 2; and ST73, 1 or 2). The mean plasmid size was 145 kb, ranging from 75 to 155 kb. One isolate contained two different plasmids encoding trimethoprim resistance (Fig. 3, lane 1). There was total agreement between PCR-based and DNA hybridization-based *dfr* gene class characterization. The hybridization experiments with the bla_{TEM} probe (not shown) revealed colocalization with *dfr* genes in all isolates but one of ST73, where the bla_{TEM} was on an ~160-kb plasmid and the *dfrA1* was on the chromosome (Fig. 3, lane 14).

Analysis of three STs that previously have been linked with antibiotic resistance and local outbreaks of urinary tract infection. The potential role of local outbreaks of urinary tract infection during the intervention was analyzed in relation to three previously reported trimethoprim-resistant and virulent "clonal groups" of *E. coli*. Antibiotic-resistant CGA, serotype O15:K52:H1, or ST131 accounted for 64/548 isolates analyzed, but there was no evident epidemiological clustering in time or in the geographical location of health care clinics providing these samples. The 26 isolates characterized as ST69 (a specific CGA subset) also possessed five different trimethoprim resistance genes (*dfrA17*, $n = 20$; *dfrA5*, $n = 3$; *dfrA7*, $n = 1$; *dfrA8*, $n = 1$; *dfrA14*, $n = 1$), signifying diverse origins for these isolates and not a recent com-

TABLE 2 Odds ratios for measuring change between 2004 and 2006 in the proportion of resistant isolates in different BAPS populations of *E. coli*

Resistance trait	BAPS population	No. of isolates with/without the resistance trait in 2006	No. of isolates with/without the resistance trait in 2004	Odds ratio[b]	*P* value
Trimethoprim resistance	BAPS 1	49/110	32/140	**1.95**	0.011
	BAPS 2	27/39	37/18	**0.32**	0.004
	BAPS 3	24/24	31/15	0.48	0.089
Trimethoprim susceptible but other resistance present[a]	BAPS 1	23/136	21/151	1.22	0.551
	BAPS 2	11/55	2/53	**5.29**	0.025
	BAPS 3	3/45	4/42	0.70	0.672
Susceptible to all tested agents	BAPS 1	87/72	119/53	**0.54**	0.007
	BAPS 2	28/38	16/39	1.80	0.132
	BAPS 3	21/27	11/35	**2.48**	0.043

[a] "Other resistance" applies to resistance to any of ampicillin, amdinocillin, cefadroxil, nitrofurantoin, or nalidixic acid.
[b] Bold text signifies statistically significant effect sizes.

mon ancestry from a single clone. Similarly, the ST131 isolates had several different trimethoprim resistance genes (*dfrA1*, $n = 1$; *dfrA17*, $n = 4$; *dfrA7*, $n = 1$; *dfrA12*, $n = 4$, *dfrA14*, $n = 1$; not determined [ND], $n = 1$). In addition to trimethoprim-resistant isolates of ST69, CGA corresponded to the closely related ST394, ST1420, and ST1451, found as single isolates in our study (Fig. 2). CGA isolates were from both years 2004 ($n = 14$) and 2006 ($n = 15$). Trimethoprim-susceptible isolates with genetic backbones matching the CGA were also present in both years. The trimethoprim-resistant serotype O15:K52:H1 corresponded to the trimethoprim-resistant isolates of ST393 found in both 2004 ($n = 3$) and 2006 ($n = 2$) and a single isolate of ST1447 found in 2006 (Fig. 2). Finally, trimethoprim-resistant isolates of ST131 were recovered in 2004 ($n = 6$) and 2006 ($n = 6$).

DISCUSSION

In this study, we investigated the *E. coli* population before and after 2 years of a prospective and pronounced reduction in the use of trimethoprim in a health care region in Sweden, where the overall resistance frequency in *E. coli* remained stable. We especially studied the distribution of *dfr* genes encoding trimethoprim resistance in relation to different *E. coli* subpopulations. The use of MLST allowed us to identify 163 STs among 548 *E. coli* isolates derived from urinary specimen cultures and to perform before-and-after analyses of the distribution of trimethoprim resistance. By the use of the BAPS software for depicting the genetic structure of *E. coli*, we identified three principal genetic subpopulations of *E. coli*, which we designated BAPS 1, BAPS 2, and BAPS 3, and analyzed them in relation to trimethoprim resistance. We found that the distributions of resistance were highly unequal among the three subpopulations and that these distributions differed before and after the reduction in the use of trimethoprim. The findings illustrate that behind a seemingly stable trimethoprim resistance frequency in *E. coli* there may be substantial underlying change in resistance frequency and distribution at the subpopulation level.

Importantly, the ST assignment into three BAPS subpopulations agreed mainly with NJ analysis, which generates a more traditional phylogenetic division of the *E. coli* population. The BAPS subpopulations corresponded largely to the *E. coli* phylogroups A and B1 (the BAPS 2 cluster), B2 (BAPS 1), and D (BAPS 3) (36, 37). We preferred to use the BAPS clustering in this work, because this clustering method takes the possibility of homologous recombination into account and the phylogroups A and B1 are genetically closely related and typically difficult to resolve using MLST data (9, 37, 38). Trimethoprim resistance was frequent in BAPS 2 and BAPS 3, while BAPS 1, consisting of a greater number of isolates, contained less trimethoprim resistance. The distributions of STs were highly similar before and after the intervention, and

TABLE 3 Number of isolates and odds ratios for measuring change between 2004 and 2006 in the proportion of trimethoprim-resistant isolates in STs of *E. coli*[b]

Genetic population	No. of isolates sampled in 2004		No. of isolates sampled in 2006		Ratio of isolate numbers in 2006/2004	*P* value	Odds ratio[a]	*P* value
	Resistant	Susceptible	Resistant	Susceptible				
ST73 (BAPS 1)	9	38	15	27	0.89	0.643	2.33	0.085
ST127 (BAPS 1)	1	21	5	5	**0.45**	0.045	**20.0**	0.007
ST141 (BAPS 1)	1	14	2	14	1.06	1	2.00	0.616
ST95 (BAPS 1)	2	12	2	9	0.79	0.682	1.33	0.762
ST12 (BAPS 1)	0	7	4	9	1.89	0.255	Plus infinity	0.073
ST131 (BAPS 1)	6	7	6	0	0.46	0.161	**Plus infinity**	0.015
ST80 (BAPS 1)	4	10	0	3	**0.21**	0.014	Approaches 0	0.288
ST10 (BAPS 2)	12	0	4	9	1.09	1	**Approaches 0**	0
ST69 (BAPS 3)	11	2	15	7	1.69	0.162	0.39	0.295
Other STs (BAPS 1)	9	31	15	43	1.46	0.058	1.20	0.71
Other STs (BAPS 2)	25	18	23	30	1.23	0.312	0.55	0.152
Other STs (BAPS 3)	20	13	9	17	0.79	0.408	**0.34**	0.048

[a] In order to calculate OR, 0.5 was added to all values when one value was 0.
[b] One isolate from year 2004 and another from 2006 did not belong in BAPS clusters 1 to 3 and hence was not included. Bold text signifies statistically significant changes.

TABLE 4 The number of trimethoprim-resistant isolates per *dfr* gene class and *E. coli* BAPS population for years 2004 and 2006

dfr gene class	No. of trimethoprim-resistant isolates					
	BAPS 1 2004	BAPS 1 2006	BAPS 2 2004	BAPS 2 2006	BAPS 3 2004	BAPS 3 2006
A1	15	25	15	7	4	2
A5	4	5	8	9	4	2
A7	0	3	2	1	1	1
A8	2	3	0	0	1	1
A12	4	2	1	3	1	1
A14	2	3	4	3	3	1
A17	2	5	3	2	17	15
Other[a]	1	0	2	1	0	0
ND[b]	2	3	2	1	0	1

[a] Other *dfr* genes detected were *dfr2d* ($n = 1$), *dfrA24* ($n = 1$), *dfrA1* and *dfrA17* ($n = 1$), and *dfrA1* and *dfrA5* ($n = 1$).
[b] ND, not determined.

we identified no dominant expansion of specific antibiotic-resistant STs in BAPS 1 that could explain the increased trimethoprim resistance level in this subpopulation after the intervention. Overall, the most common STs were ST73, ST69, ST127, ST141, ST10, ST95, ST12, ST131, or ST80. The importance of these STs as UTI-causing lineages is supported by the finding of a similar distribution of STs in a recent study from North West England (39). Similarly, a multinational survey of UTI in 16 European countries and Canada using phenotyping of 2,482 *E. coli* isolates identified a predominance of only a few *E. coli* types (40). A corresponding pattern was also observed in a recent MLST analysis of 220 *E. coli* isolates causing bloodstream infection, where five genotypes accounted for two-thirds of the isolates (41). The observation in this work of an association between antimicrobial resistance levels and BAPS subpopulations of *E. coli* is in line with that of other investigators, who, by applying multiplex PCR on 1,533 blood isolates, recently showed an association with phylogroups (42). Although we cannot deduce the mechanisms behind the observation by analyzing our data, a plausible reason has been demonstrated by other workers performing multiple genome comparisons of *E. coli* (38, 43). Their work supports the hypothesis that there is a preferred way of gene sharing within phylogroups, including evidence that interphylogroup gene flow is lower than intraphylogroup gene flow, which is compatible with the fact that antibiotic resistance genes are shared within phylogroups more frequently than between phylogroups.

The association between resistance and genetic subpopulation was marked on the ST level, where some STs contained high frequencies of trimethoprim-resistant isolates, e.g., ST69, ST405, ST88, ST58, and ST10. In contrast, other STs (e.g., ST95, ST127, and ST141) contained low frequencies of resistant isolates. A current paradigm is that successful virulent clones of *E. coli* associated with antibiotic resistance can emerge rapidly within the population through clonal expansion and thereby become locally (44–46) or even globally successful (16, 19, 47–49). Our findings, however, of both resistant and susceptible isolates of the same STs in the local setting suggest a more complex scenario. The carriage of resistance in particular STs seems not to be a clonal phenomenon at the ST level. Detailed analysis of the chromosomal or plasmid location of *dfr* genes in isolate subsets of ST69, ST58, and ST73 showed patterns indicative of multiple parallel acquisitions or an extensive exchange of resistance genes between different plasmids or, occasionally, the chromosome. A straightforward interpretation of our data would be that the concept of "epidemic antibiotic-resistant clones" or "high-risk antibiotic-resistant clones" when defining a clone by the ST concept is too unspecific. Indeed, recent analyses of ST131 by whole-genome analysis show that more detailed analysis than provided by MLST is required to identify true clones of *E. coli* and that even with genome-wide typing resolution, a history of multiple resistance gene acquisitions cannot be excluded (19). Complementary to the concept of clonal spread of antibiotic resistance, we therefore suggest that our results partially can be explained by a rapid dissemination of antibiotic resistance genes among strains of the same or a closely related ST by horizontal transfers. This model of resistance spread would also explain why we observed so many different trimethoprim resistance gene classes in some STs.

Despite the overall stable resistance rates, genetic diversity, and distribution of genetic subpopulations, distinct changes of antibiotic resistance rates were identified at the *E. coli* subpopulation level in this study. Comparing the first and second time period studied, the proportion of trimethoprim resistance increased in BAPS 1 and decreased in BAPS 2. In parallel, the proportion of *E. coli* susceptible to all six investigated antibiotics decreased in BAPS 1 and increased in BAPS 2 and BAPS 3. We cannot explain why resistance would move from parts of the population with initially high levels of resistance (BAPS 2 and BAPS 3) to parts with a lower resistance level (BAPS 1), but possibly the three subpopulations have different abilities to cope with change of antibiotic pressures. Notably, only in BAPS 2 a decrease in trimethoprim resistance was observed between the two time periods. Interestingly BAPS 2 also gained resistance to other antibiotics, a shift which coincided with the changes in antibiotic prescription during the intervention from a common use of trimethoprim to the use of other antimicrobials (29).

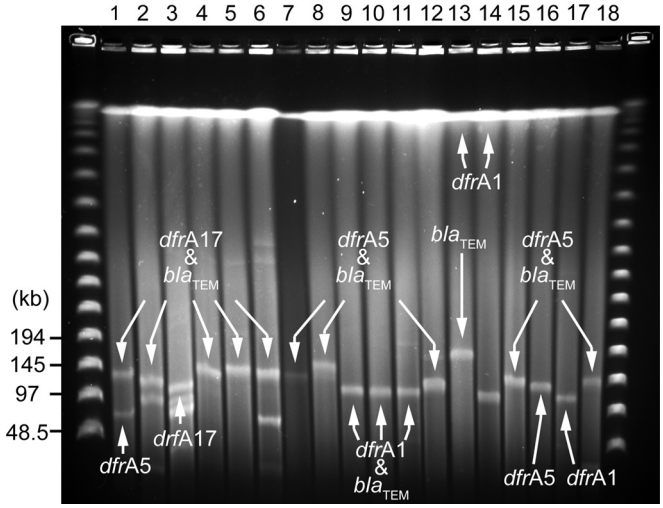

FIG 3 Pulsed-field gel electrophoresis of S1-digested DNA to identify plasmid or chromosomal localizations of antibiotic resistance genes in *E. coli* isolates. Isolates of ST69 (lanes 1 to 6), ST58 (lanes 7, 8, 12, 15, 16, 18), and ST73 (lanes 9 to 11, 13, 14, 17) are shown. The banding pattern of a gel lane corresponds with plasmid sizes present in an isolate. The chromosomal DNA consistently shows as the uppermost band. Size standards are found at the left and right. The localization of trimethoprim resistance genes (*dfrA1*, *dfrA5*, *dfrA17*) and the beta-lactamase gene (bla_{TEM-1}) is indicated by arrows.

A speculative explanation would be an increased adaptability of BAPS 2 to the changed antibiotic pressures compared with other subpopulations. We acknowledge that our study design due to costs lacked a control area without an intervention, reducing the use of trimethoprim. Thus, we cannot exclude the possibility that the within-population changes of *E. coli* observed between the two time periods would have been the same in a control area. It is clear, though, that the differences observed between the two time periods are striking and that resistance gene frequencies within the *E. coli* population changed much beyond what can be explained by an increase/decrease of any of the previously described antibiotic-resistant *E. coli* clones with a worldwide spread.

The increase in trimethoprim resistance in BAPS 1 observed during the second time period was almost entirely caused by an increase in the presence of *dfrA1* with a coinciding decrease in the prevalence of this gene in BAPS 2 and 3. The process resembles a diffusion process in which the frequency of resistance is equalized between the subpopulations. We speculate that since the *dfrA1* is the first and the most common *dfr* gene reported in the literature, evolution may have had sufficient time to develop mechanisms facilitating spread within the whole *E. coli* population. We found that *dfrA1* is well adapted to and widely spread in the entire *E. coli* population and that it can be located both on the chromosome and on several plasmids. In contrast, the distribution of other *dfr* genes (e.g., *dfrA5* in ST58 and *dfrA17* in ST69) seems more restricted, maybe because the genes themselves or accessory sequences are not yet evolutionary fine-tuned to fit the entire population.

The statistical significance testing in this study was not corrected for multiple comparisons. We acknowledge that such analysis, e.g., using Bonferroni correction, would decrease the number of significant results. Multiple comparison correction, however, may be too conservative to be used in the present setting. While controlling for false positives as intended, multiple testing comes at the cost of increasing the number of false negatives. We estimate that less than 14% of our results presented as significant are false positives, because 64 hypotheses were tested (all presented in the paper) and 24 positives resulted ($P < 0.05$). In a fictive worst-case scenario with regard to false positives, all the null hypotheses tested were in reality true, and we have obtained approximately 3.2 false positives (given some assumptions about the distribution of the data). Since 24 positive results were obtained, this suggests that only a small fraction (<14%) are false positives. This is an overestimation, because it is unlikely given the observed data that all the null hypotheses were true and that there were no false negatives, and we consequently preferred to present the data without multiple corrections.

In conclusion, the data presented here obtained using a before-and-after study design in relation to a large-scale community intervention on the use of trimethoprim revealed large changes in the distribution of trimethoprim resistance in different subpopulations of *E. coli*. The changes occurred despite a stable overall resistance level in the population as a whole. Interestingly, we found little support that the within-population changes were caused by inflow or outflow of any of the previously described antibiotic-resistant "high-risk clones" of *E. coli*. There was, however, clear patterns of association of *dfr* genes with genetic subpopulations. The results presented in this work provide limited mechanistic explanations and cannot unambiguously determine the role of the intervention but are compatible with a scenario of "within-population relocation" of antibiotic resistance genes from a subpopulation connected with high resistance levels before the intervention (BAPS 2) to another subpopulation with lower resistance levels after the intervention (BAPS 1). The observations emphasize the complexity of antibiotic resistance dissemination in *E. coli*.

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