Molecular Characterisation of Trimethoprim Resistance in *Escherichia coli* and *Klebsiella pneumoniae* during a Two Year Intervention on Trimethoprim Use

Alma Brolund¹*, Martin Sundqvist², Gunnar Kahlmeter³,4, Malin Grape¹

¹Department of Microbiology, Tumor and Cell Biology (MTC), Division of Clinical Microbiology, Karolinska Institutet, Stockholm, Sweden, ²Department of Clinical Microbiology, Central Hospital, Växjö, Sweden, ³Division of Infectious Diseases, Department of Medical Sciences, Uppsala University, Uppsala, Sweden, ⁴Division of Clinical Bacteriology, Department of Medical Sciences, Uppsala University, Uppsala, Sweden

Abstract

**Background:** Trimethoprim resistance is increasing in *Enterobacteriaceae*. In 2004-2006 an intervention on trimethoprim use was conducted in Kronoberg County, Sweden, resulting in 85% reduction in trimethoprim prescriptions. We investigated the distribution of dihydrofolate reductase (dfr)-genes and integrons in *Escherichia coli* and *Klebsiella pneumoniae* and the effect of the intervention on this distribution.

**Methodology/Principal Findings:** Consecutively isolated *E. coli* (n = 320) and *K. pneumoniae* (n = 54) isolates phenotypically resistant to trimethoprim were studied. All were investigated for the presence of dfrA1, dfrA5, dfrA7, dfrA8, dfrA12, dfrA14, dfrA17 and integrons class I and II. Isolates negative for the seven dfr-genes (n = 12) were also screened for dfrA2d, dfrA3, dfrA9, dfrA10, dfrA24 and dfrA26. These genes accounted for 96% of trimethoprim resistance in *E. coli* and 69% in *K. pneumoniae*. The most prevalent was dfrA1 in both species. This was followed by dfrA17 in *E. coli* which was only found in one *K. pneumoniae* isolate. Class I and II Integrons were more common in *E. coli* (85%) than in *K. pneumoniae* (57%). The distribution of dfr-genes did not change during the course of the 2-year intervention.

**Conclusions/Significance:** The differences observed between the studied species in terms of dfr-gene and integron prevalence indicated a low rate of dfr-gene transfer between these two species and highlighted the possible role of narrow host range plasmids in the spread of trimethoprim resistance. The stability of dfr-genes, despite large changes in the selective pressure, indirectly suggests a low fitness cost of dfr-gene carriage.

Introduction

The increase in antibiotic resistance is a threat to global health [1]. Trimethoprim is commonly used in the treatment of urinary tract infections (UTI) in all parts of the world [2]. However, already soon after the introduction of the drug, trimethoprim resistance was reported in several species [3] and are now in unselected UTI materials at levels of 15–65% in *E. coli* [4,5,6]. This leads to treatment failure and increasing workload in primary healthcare and trimethoprim containing antibiotics are thus questioned as first line therapy [7].

Resistance to trimethoprim is caused by modifications in the target enzyme dihydrofolate reductase (dfr) encoded by dfr-genes. So far 30 dfr-genes are described and they are usually associated with integrons [3]. Integrons are integrated in transposons predominantly located on plasmids and can insert, excise and express mobile gene cassettes, often antibiotic resistance genes [8]. This results in an efficient horizontal spread of antibiotic resistance between bacteria [8,9,10]. Only few studies have investigated the epidemiology and frequency of the different dfr-genes and the relationship to integrons and other resistance determinants in *E. coli* [9,11,12]. In *K. pneumoniae* the distribution of dfr-genes is not known.

A two year prospective intentional intervention on the use of trimethoprim was performed in Kronoberg County, Sweden, from October 1st, 2004 to September 30th, 2006. A drastic and sustained 85% reduction in the use of trimethoprim and trimethoprim-sulphamethoxazole was achieved at county level. A corresponding increase was seen in the use of nitrofurantoin, pivmecillinam and to some extent ciprofloxacin. The effect on trimethoprim resistance was marginal [13] explained by the low fitness cost of trimethoprim resistance observed and the high level of associated resistance in trimethoprim resistant isolates [13]. During 4 years including the intervention all urinary tract isolates of *Enterobacteriaceae*, both hospital and community acquired, were stored frozen at −70°C. Using these, we investigated the distribution of 13 dfr-genes and class I and II integrons in trimethoprim resistant *E. coli* and *K. pneumoniae*. We also investigated the associated resistance rates in these isolates and
the findings are discussed in relation to the epidemiology of trimethoprim resistance within and between the two species.

Materials and Methods

Material

Three hundred and twenty (320) consecutively collected trimethoprim resistant E. coli isolates during three time periods were identified. The periods were: four months before the intervention (June to September 2004 (n = 106), one year into the intervention (October to December 2005 (n = 105)) and the last four months of the intervention (June to September 2007 (n = 109)). Within each of the time periods duplicate patient samples were excluded. Fifty four (54) consecutive trimethoprim resistant E. coli isolates from June 2004 to September 2007, reflecting the relative number of isolates of the respective pathogens in UTI, were retrieved. Isolates were from urinary samples from hospitals and community and from all age groups.

Methods

Susceptibility testing. Escherichia coli and K. pneumoniae were identified according to standard procedures used in the Department of Clinical Microbiology, Vaxjö, Sweden, at the time of the study. Susceptibility testing was performed using disc diffusion on IsoSensitest Agar (Oxoid, Basingstokes, UK) according to the Swedish Reference Group on Antibiotics (SRGA) guidelines (http://www.srgba.org/). All isolates were tested with the following antibiotic discs (Oxoid, Basingstokes, UK) mecillinam 10 μg, ampicillin 10 μg, cefadroxil 30 μg, trimethoprim 5 μg, nitrofurantoin 100 μg and nalidixic acid 30 μg. To obtain the most sensitive detection of antibiotic resistance, the resistant and susceptible populations were identified by calculating the epidemiological cut-off values by using the Normalized interpretation method [NRI] [14]. For mecillinam the SRGA R-breakpoint was used at the time of isolation was applied to avoid false associations to TMP resistance due to concomitant AMP resistance affecting the MEC MIC only marginally (i.e. MIC≤8) and considered not to be due to specific mecillinam resistance mechanisms (Kahlmeter unpublished data). Trimethoprim resistance was further analysed using E-test (bioMérieux, Solna, Sweden).

PCR. DNA lysates were prepared by resolving a loop full (1 μl) of bacteria in 100 μl DNase and RNase free water (Sigma-Aldrich, Stockholm, Sweden). The mixture was boiled for 2 minutes and the tubes were centrifuged at 13000 rpm for 2 minutes. The supernatant was stored in −20°C and used as DNA template. All isolates, both E. coli and K. pneumoniae, were first screened for the five most prevalent resistance genes reported in trimethoprim resistant E. coli; dfrA1, dfrA5, dfrA7, dfrA12 and dfrA17 [9,11] using a previously published real-time multiplex PCR protocol [15]. In addition dfrA8 and dfrA14 was screened for among all isolates. In isolates negative to these genes (n = 12) dfr2d, dfrA3, dfrA9, dfrA10, dfrA24 and dfrA26 were analysed using separate PCRs. Internal controls with primers for 16S rDNA was used as template control [16]. All isolates were in addition analyzed for the presence of integron class I and II, which are the most common integrons associated with dfr-genes [8]. The screening of dfr-genes and integrons class I and II was performed using simplex PCRs, described by Grape et al [17]. The PCR for integron class I detection was performed using two sets of primers to avoid false negative results due to modifications in the 3’CS region. A PCR product with either of these primer pairs was considered a positive result. Previously unpublished primers are described in Table 1.

Semi-random PCR. Semi random PCR [18] was performed on three E. coli isolates where the PCR only yielded an amplicon corresponding to the 5’CS (IntI1) of integron class I. Primers for identification of resistance cassettes integrated in integron class I were designed targeted at IntI1; 51 bp and 27 bp respectively from the cassette region (Table 2).

Sequence analysis. Sequence analysis was performed on the semi-random PCR amplicons as well as on two isolates where we could only detect a complete integron class I structure (5’CS-3’CS). PCR products (50 μl reactions) were purified using Jet Quick PCR Product Purification Spin kit (Genomed, Lohne, Germany) or, in case of unspecified PCR products, extracted from electrophoresis gel by using the Jet Quick Gel Extraction Spin kit (Genomed, Lohne, Germany). Sequencing reactions were performed by using the ABI Prism Big Dye Terminator Cycle Sequencing kit v. 3.1 (Applied Biosystems, Stockholm, Sweden) according to manufacturer’s instructions. Sequence data was analysed with the Sequencher software v. 4.1.4 (Gene Codes Corporation, Ann Arbor, MI).

Ethics

As this study was based on bacterial isolates collected from routine samples, approval from ethics committee was not necessary. Neither was there any need for informed consent procedures as no extra sampling was performed and no personal data was stored in relation to the isolates.

Statistics

χ²-test was used for comparing dfr-gene prevalence and distribution in E. coli and K. pneumoniae.

Results

dfr-Genes and Integrons

The total prevalence of the 13 analysed dfr-genes was 96% (n = 308) in E. coli and 69% (n = 38) in K. pneumoniae. The integron

| Table 1. Primers not previously published used in the PCR screenings. |
| --- |
| Primer | Locus | Sequence (5’-3’) | Annealing temperature (C) | Product size (bp) | Reference |
| dfrA14-f | 5’ dfrA14 | CTG CGA AAG CGA AAA ACG GCG | 55 | 376 | This study |
| dfrA14-r | 3’ dfrA14 | GGA ATA CTC GGG AAG AAA ACA | 55 | 190 | This study |
| dfrA24-f | 5’ dfrA24 | CGT TGC TGC TAC TGA GAA CG | 54 | 158 | This study |
| dfrA24-r | Mid dfrA24 | TGC GGT CTT TCA GAG GAC TT | 54 | 190 | This study |
| dfrA26-f | 5’ dfrA26 | GGT AAC GGC TCA ACA AGG TT | 56 | 190 | This study |
| dfrA26-r | 3’ dfrA26 | GGC GTG TAC TTC GTG GAG AT | 56 | 190 | This study |

doi:10.1371/journal.pone.0009233.t001
prevalence (both integron class I and II) was 85% (n = 273) and 57% (n = 51) in E. coli and K. pneumoniae, respectively. These differences were statistically significant (p<0.001). The most common trimethoprim resistance gene in both investigated species was dfrA1. The second most frequent dfr-gene in E. coli, dfrA17, was rare in K. pneumoniae - it was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.

The prevalence of each of the 13 different dfr-genes and integrons class I and II found in the E. coli and K. pneumoniae isolates are presented in Table 3. No major shifts in the dfr-gene/integron distribution were seen in the E. coli isolates during the intervention. The K. pneumoniae collection was too small for statistically significant changes to be detected.

In 83 E. coli isolates the integron screenings only detected the integrase gene (5’CS class 1 integrons 51 bp from cassette region). The most was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.

In 83 E. coli isolates the integron screenings only detected the integrase gene (5’CS class 1 integrons 51 bp from cassette region). The most common trimethoprim resistance gene in both investigated species was dfrA1. The second most frequent dfr-gene in E. coli, dfrA17, was rare in K. pneumoniae - it was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.

The prevalence of each of the 13 different dfr-genes and integrons class I and II found in the E. coli and K. pneumoniae isolates are presented in Table 3. No major shifts in the dfr-gene/integron distribution were seen in the E. coli isolates during the intervention. The K. pneumoniae collection was too small for statistically significant changes to be detected.

In 83 E. coli isolates the integron screenings only detected the integrase gene (5’CS class 1 integrons 51 bp from cassette region). The most common trimethoprim resistance gene in both investigated species was dfrA1. The second most frequent dfr-gene in E. coli, dfrA17, was rare in K. pneumoniae - it was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.

The prevalence of each of the 13 different dfr-genes and integrons class I and II found in the E. coli and K. pneumoniae isolates are presented in Table 3. No major shifts in the dfr-gene/integron distribution were seen in the E. coli isolates during the intervention. The K. pneumoniae collection was too small for statistically significant changes to be detected.

In 83 E. coli isolates the integron screenings only detected the integrase gene (5’CS class 1 integrons 51 bp from cassette region). The most common trimethoprim resistance gene in both investigated species was dfrA1. The second most frequent dfr-gene in E. coli, dfrA17, was rare in K. pneumoniae - it was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.

The prevalence of each of the 13 different dfr-genes and integrons class I and II found in the E. coli and K. pneumoniae isolates are presented in Table 3. No major shifts in the dfr-gene/integron distribution were seen in the E. coli isolates during the intervention. The K. pneumoniae collection was too small for statistically significant changes to be detected.

In 83 E. coli isolates the integron screenings only detected the integrase gene (5’CS class 1 integrons 51 bp from cassette region). The most common trimethoprim resistance gene in both investigated species was dfrA1. The second most frequent dfr-gene in E. coli, dfrA17, was rare in K. pneumoniae - it was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.

The prevalence of each of the 13 different dfr-genes and integrons class I and II found in the E. coli and K. pneumoniae isolates are presented in Table 3. No major shifts in the dfr-gene/integron distribution were seen in the E. coli isolates during the intervention. The K. pneumoniae collection was too small for statistically significant changes to be detected.

In 83 E. coli isolates the integron screenings only detected the integrase gene (5’CS class 1 integrons 51 bp from cassette region). The most common trimethoprim resistance gene in both investigated species was dfrA1. The second most frequent dfr-gene in E. coli, dfrA17, was rare in K. pneumoniae - it was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.

The prevalence of each of the 13 different dfr-genes and integrons class I and II found in the E. coli and K. pneumoniae isolates are presented in Table 3. No major shifts in the dfr-gene/integron distribution were seen in the E. coli isolates during the intervention. The K. pneumoniae collection was too small for statistically significant changes to be detected.

In 83 E. coli isolates the integron screenings only detected the integrase gene (5’CS class 1 integrons 51 bp from cassette region). The most common trimethoprim resistance gene in both investigated species was dfrA1. The second most frequent dfr-gene in E. coli, dfrA17, was rare in K. pneumoniae - it was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.

The prevalence of each of the 13 different dfr-genes and integrons class I and II found in the E. coli and K. pneumoniae isolates are presented in Table 3. No major shifts in the dfr-gene/integron distribution were seen in the E. coli isolates during the intervention. The K. pneumoniae collection was too small for statistically significant changes to be detected.

In 83 E. coli isolates the integron screenings only detected the integrase gene (5’CS class 1 integrons 51 bp from cassette region). The most common trimethoprim resistance gene in both investigated species was dfrA1. The second most frequent dfr-gene in E. coli, dfrA17, was rare in K. pneumoniae - it was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.

The prevalence of each of the 13 different dfr-genes and integrons class I and II found in the E. coli and K. pneumoniae isolates are presented in Table 3. No major shifts in the dfr-gene/integron distribution were seen in the E. coli isolates during the intervention. The K. pneumoniae collection was too small for statistically significant changes to be detected.

In 83 E. coli isolates the integron screenings only detected the integrase gene (5’CS class 1 integrons 51 bp from cassette region). The most common trimethoprim resistance gene in both investigated species was dfrA1. The second most frequent dfr-gene in E. coli, dfrA17, was rare in K. pneumoniae - it was found in only one isolate. The genes dfrA2 and dfrA24 were found only in E. coli and genes dfrA3 and dfrA9 only in K. pneumoniae. Four E. coli isolates and one K. pneumoniae isolate carried two dfr-genes. The dfrA1 gene was present together with either dfrA5, dfrA7, dfrA14 or dfrA17 in E. coli and together with dfrA8 in K. pneumoniae.
resistance was 26% in trimethoprim resistant isolates [19]. In the present material ampicillin resistance was 78% and nalidixic acid resistance in other species than E. coli has not been well studied at narrow host range plasmids, or are integrated in the chromosome. [20]. Multiple dfr-genes occur but are unusual. The prevalence of dfr-genes in two out of 163 trimethoprim resistant isolates detected coexistence of two genes in four out of 320 E. coli isolates and one out of 54 K. pneumoniae isolates. Previous studies also confirm this; in a large European study [9] they found multiple dfr-genes in two out of 163 trimethoprim resistant isolates and in a Korean study [11] the same figure was three out of 77 trimethoprim resistant isolates.

### Discussion

Although common, the epidemiology of trimethoprim resistance in other species than E. coli has not been well studied at the molecular level. Recently we performed a highly controlled intervention on trimethoprim use in Kronoberg County, Sweden. Despite a drastic decrease (85%) in trimethoprim consumption the effect on the rate of trimethoprim resistance in E. coli was disappointingly marginal [13]. This was explained by co-selection mediated by a high level of associated resistance in trimethoprim resistant isolates and by a low fitness cost conferred by trimethoprim resistance measured in vitro in clinical isolates of E. coli [13]. The clonal distribution in E. coli remained unaffected [13]. The present study shows a lack of effect on the overall distribution of dfr-genes (Table 3). It also compares the distribution of dfr-genes in E. coli and K. pneumoniae.

The overall prevalence of the different dfr-genes in E. coli was in line with earlier studies performed in different clinical and geographical settings by, amongst others, Blahna [9] and Lee. [11] These studies have indicated a very stable distribution of dfr-genes over time with dfaA1 and dfaA17 as the most prevalent genes. The stability over time and throughout the intervention suggests that the epidemiological fitness cost of the most common dfr-genes or of plasmids carrying these genes is very low. The fact that dfaA8 and dfaA14 were as common as dfaA7 and dfaA12 indicates possible future shifts in the dfr-gene distribution. So far dfaA2, dfaA8, dfaA9, dfaA10 and dfaA24 have not been reported as cassettes in integron structures [20]. Multiple dfr-genes occur but are unusual. The PCR screening performed for dfaA1, dfaA5, dfaA7, dfaA8, dfaA12, dfaA14 and dfaA17 detected coexistence of two genes in four out of 320 E. coli isolates and one out of 54 K. pneumoniae isolates. Previous studies also confirm this; in a large European study [9] they found multiple dfr-genes in two out of 163 trimethoprim resistant isolates and in a Korean study [11] the same figure was three out of 77 trimethoprim resistant isolates. Based on this we decided to screen for the additional six dfr-genes in the trimethoprim resistant isolates that were negative in the multiplex PCR analysis.

The prevalence of dfr-genes and integrons in K. pneumoniae has not been studied systematically before and was surprisingly different from E. coli. The low prevalence of integrons class I and II could suggest other mechanisms at play or that transfer of dfr-genes in K. pneumoniae does not occur. The genes dfaA5, dfaA8 and dfaA12 were more common in K. pneumoniae than in E. coli and dfaA17, being the second most frequent dfr-gene in E. coli, was only found in a single K. pneumoniae isolate. Recent studies in mice showed that plasmids carrying varying resistance genes were easily transferred from an E. coli donor to K. pneumoniae in a mouse model. [21] Our data, showing a pronounced disproportion in the prevalence of integrons and dfr-genes in E. coli and K. pneumoniae, speak to the opposite. This could be because dfr-genes are carried on narrow host range plasmids, or are integrated in the chromosome.

### Table 4. The relationship between dfr-genes and integron class I and II in E. coli (n = 320) and K. pneumoniae (n = 54).

| E. coli | No. | No. | K. pneumoniae | No. | No. |
|--------|-----|-----|---------------|-----|-----|
|        | Igr1 | Igr2 |               | Igr1 | Igr2 |
| dfaA1* | 61   | 44  | dfaA1*        | 5    | 2   |
| dfaA2  | 0    | 0   | dfaA2         | 0    | 0   |
| dfaA3  | 0    | 0   | dfaA3         | 1    | 0   |
| dfaA5  | 49   | 0   | dfaA5         | 7    | 0   |
| dfaA7  | 15   | 0   | dfaA7         | 1    | 0   |
| dfaA8  | 1    | 0   | dfaA8         | 1    | 0   |
| dfaA9  | 0    | 0   | dfaA9         | 0    | 0   |
| dfaA10 | 0    | 0   | dfaA10        | 0    | 0   |
| dfaA12 | 12   | 0   | dfaA12        | 7    | 0   |
| dfaA14 | 8    | 0   | dfaA14        | 4    | 0   |
| dfaA17 | 81   | 0   | dfaA17        | 1    | 0   |
| dfaA24 | 1    | 0   | dfaA24        | 0    | 0   |
| dfaA26 | 0    | 0   | dfaA26        | 0    | 0   |
| All dfr+ | 228  | 44  | All dfr+      | 27   | 2   |

*aIncluding 10 isolates positive for both Igr1 and 2.

### Table 5. No. of E. coli (n = 320) isolates resistant to trimethoprim only and in combination with other antibiotics and in relation to the presence of integrons.

| Resistance phenotype | isolates (#) | Integron (%) |
|----------------------|--------------|--------------|
| TMP (only)           | 58           | 71           |
| TMP, AMP             | 66           | 61           |
| TMP, NAL             | 13           | 69           |
| TMP, AMP, NAL        | 56           | 91           |
| TMP, AMP, MEC        | 10           | 90           |
| TMP, AMP, CFR        | 3            | 100          |
| TMP, NAL, CFR        | 1            | 100          |
| TMP, AMP, NAL, CFR   | 8            | 100          |
| TMP, AMP, NAL, CFR, NIT | 5   | 80           |
| TMP, AMP, NAL, MEC   | 1            | 100          |

| Resistance phenotype | isolates (#) | Integron (%) |
|----------------------|--------------|--------------|
| TMP, AMP, NIT, MEC   | 27           | 67           |
| TMP, AMP, NIT, MEC, NAL | 18   | 39           |
| TMP, AMP, NIT, MEC, NAL, CFR | 1  | 100          |
| TMP, AMP, NIT, MEC, NAL, CFR, NIT | 1 | 100          |

TMP = trimethoprim, AMP = ampicillin, NAL = nalidixic acid, MEC = mecillinam, CFR = cefadroxil and NIT = nitrofurantoin.

doi:10.1371/journal.pone.0009233.t005

doi:10.1371/journal.pone.0009233.t006

doi:10.1371/journal.pone.0009233.t007
mosomal DNA or because of differences in fitness cost of specific dfr-genes in *E. coli* and *K. pneumoniae*. The trimethoprim resistant *K. pneumoniae* were collected over three years, from individuals of all age groups and varying residency. The *K. pneumoniae* isolates were not typed but with this background clonal spread of these isolates seems an unlikely explanation for the observed differences between *E. coli* and *K. pneumoniae*.

Trimethoprim resistance is often associated with other resistance determinants. [8,9,17,22] This gives the possibility for co-selection of trimethoprim resistant strains and plasmids carrying dfr genes as well as other resistance determinants by the use of other antibiotic classes. In this study no striking difference in the distribution of other resistance determinants in relation to specific dfr-genes was seen. The only exception was *E. coli* isolates carrying dfrA17 (n = 10) where an association exclusively with ampicillin resistance were found. Only one out of ten of these isolates carried an integron class I. As a bright contrast, resistance were found. Only one out of ten of these isolates carried an integron class I. This indicates that the exchange of dfr-genes between *E. coli* and *K. pneumoniae* may be an uncommon event in man. These results suggest the need for more studies on the genetic context of dfr-genes and their location in or association with mobile genetic elements and other resistance genes as well as their association to certain plasmid types. It reminds us that we can not only rely on model organisms, such as *E. coli*, to understand the variety of phenotypic resistance.

### Acknowledgments

Professor Dan I Andersson, Dr. Linus Sandgren and PhD stud. Sanna Koskiniemi for introducing us to the semi-random PCR method. Co-workers at the laboratory in Växjö, especially Stina Bengtsson, Maria Sjöholm-Karlsson (presently at the CDC, Atlanta) and Gunilla Cederbrant.

### Author Contributions

Conceived and designed the experiments: AB MBS GK MG. Performed the experiments: AB MG. Analyzed the data: AB MBS MG. Contributed reagents/materials/analysis tools: MBS GK MG. Wrote the paper: AB MBS GK MG.

### References

1. Wise R, Hart T, Cars O, Straves M, Helmuth R, et al. (1998) Antimicrobial resistance. Is a major threat to public health. Br J Clin Pract 317: 609–610.

2. Huovinen P, Sundstrom L, Swedberg G, Skold O (1995) Trimethoprim and sulfonamide resistance. Antimicrob Agents Chemother 39: 279–289.

3. Skold O (2001) Resistance to trimethoprim and sulfonamides. Vet Res 32: 261–273.

4. Akram M, Shahid M, Khan AU (2007) Etiology and antibiotic resistance patterns of community-acquired urinary tract infections in J N M C Hospital Alligarh, India. Ann Clin Microbiol Antimicrob 6: 4.

5. Randrianirina F, Soares JL, Carod JR, Ratsima E, Thouinier V, et al. (2007) Antimicrobial resistance among uropathogens that cause community-acquired urinary tract infections in Antananarivo, Madagascar. J Antimicrob Chemother 59: 309–312.

6. Olson-Lidejquist B, Struve J, edi (2008) Sweden 2008 - A report on Swedish Antimicrobial Utilisation and Resistance in Human Medicine. Stockholm: Strama; the Swedish Strategic Program against Antibiotic Resistance and Swedish Institute for Infectious Disease Control.

7. Butler CC, Hillier S, Roberts Z, Dunstan F, Howard A, et al. (2006) Antibiotic-resistant infections in primary care are symptomatic for longer and increase workload: outcomes for patients with *E. coli* UTIs. Br J Gen Pract 56: 686–692.

8. White PA, Mclver CJ, Rawlinson WD (2001) Integrons and gene cassettes in the enterobacteriaceae. Antimicrob Agents Chemother 45: 2658–2661.

9. Blahna MT, Zalewski CA, Reuer J, Kahlmeter G, Foxman B, et al. (2006) The role of horizontal gene transfer in the spread of trimethoprim-sulfamethoxazole resistance among uropathogenic *Escherichia coli* in Europe and Canada. J Antimicrob Chemother 57: 666–672.

10. Dionisio F, Matic I, Radman M, Rodrigues OR, Taddei F (2002) Plasmids from Klebsiella pneumoniae to Escherichia coli in the mouse intestine. J Antimicrob Chemother 50: 513–516.

11. Kerrn MB, Klemmensen T, Frimodt-Moller N, Espersen F (2002) Susceptibility of Danish Escherichia coli strains isolated from urinary tract infections and bacteremia, and distribution of sul genes conferring sulphonamide resistance. J Antimicrob Chemother 50: 193–202.

12. Gape M, Morak S, Pavuluri S, Kahlmeter G (2007) Standard and real-time multiplex PCR methods for detection of trimethoprim resistance dfr genes in large collections of bacteria. Clin Microbiol Infect 13: 1112–1118.

13. Sundqvist M, Geli P, Andersson DI, Sjolund-Karlsson M, Runehagen A, et al. (2007) Integrons and gene cassettes in clinical isolates of co-trimoxazole-resistant Gram-negative bacteria. Clin Microbiol Infect 11: 185–192.

14. Jonsson J, Rylander M, Galau MG, Carlos C, Krounval G (2003) Analysis of parameters and validation of method for normalized interpretation of antimicrobial resistance. Antimicrob Agents Chemother 47: 525–533.

15. Gape M, Morak S, Pavuluri S, Kahlmeter G (2007) Standard and real-time multiplex PCR equalization of trimethoprim resistance dfr genes in large collections of bacteria. Clin Microbiol Infect 13: 1112–1118.

16. Kahlmeter G, Menday P (2003) Cross-resistance and associated resistance in Danish Escherichia coli strains isolated from urinary tract infections and bacteremia, and distribution of sul genes conferring sulphonamide resistance. J Antimicrob Chemother 50: 513–516.

17. Gape M, Farra A, Krounval G, Sundstrom L (2005) Integrons and gene cassettes in clinical isolates of co-trimoxazole-resistant Gram-negative bacteria. Clin Microbiol Infect 11: 185–192.

18. Nilsson AI, Koskiniemi S, Eriksson S, Kogeborg E, Hinton JG, et al. (2005) Bacterial genome size reduction by experimental evolution. Proc Natl Acad Sci U S A 102: 12112–12116.

19. Kahlmeter G, Menday P (2003) Cross-resistance and associated resistance in *E. coli* isolates from the Pan-European ECOSENS Project surveying the antimicrobial susceptibility of pathogens from uncomplicated urinary tract infections. J Antimicrob Chemother 52: 129–131.

20. Gape M, Sundstrom L, Krounval G (2007) Two new dfr genes in trimethoprim-resistant integrase-negative Escherichia coli isolates. Antimicrob Agents Chemother 51: 1863–1864.

21. Schorrning S, Struve C, Krogfelt KA (2008) Transfer of antimicrobial resistance plasmids from *Klebsiella pneumoniae* to *Escherichia coli* in the mouse intestine. J Antimicrob Chemother 62: 1098–1093.

22. Kedler K, Schwarz S (2008) Analysis and distribution of class 1 and 2 integrons and associated gene cassettes among *Escherichia coli* isolates from swine, horses, cats and dogs collected in the BfT-GermVet monitoring study. J Antimicrob Chemother 62: 469–473.

23. Kedler K, Schwarz S (2008) Analysis and distribution of class 1 and 2 integrons and associated gene cassettes among *Escherichia coli* isolates from swine, horses, cats and dogs collected in the BfT-GermVet monitoring study. J Antimicrob Chemother 62: 469–473.