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Factors that cause trimethoprim resistance in Streptococcus pyogenes
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*Streptococcus pyogenes*

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Abstract

The use of trimethoprim in treatment of *Streptococcus pyogenes* infections has long been discouraged because it was widely believed that this pathogen is resistant to this antibiotic. To gain more insight into the extent and molecular basis of trimethoprim resistance in *S. pyogenes* we have tested isolates from India and Germany and sought for the factors that conferred the resistance. Resistant isolates were identified in tests for trimethoprim or trimethoprim/sulfamethoxazole (SXT) susceptibility. Resistant isolates were screened for known horizontally transferable trimethoprim insensitive dihydrofolate reductase (*dfr*) genes *dfr*G, *dfr*F, *dfr*A, *dfr*D and *dfr*K. The nucleotide sequence of the intrinsic *dfr* was determined for resistant isolates lacking the horizontally transferable genes. Based on tentative criteria 69 out of 268 isolates (25.7%) from India were resistant to trimethoprim. Occurring in 42 of the 69 resistant isolates (60.9%) *dfr*F was more frequent than *dfr*G (23 isolates, 33.3%) in India. The *dfr*F-gene was also present in a collection of SXT-resistant isolates from Germany, in which it was the only detected trimethoprim resistance factor. *Dfr*F caused resistance in 4 out of 5 trimethoprim resistant isolates from the German collection. An amino acid substitution in the intrinsic dihydrofolate reductase known from trimethoprim resistant *Streptococcus pneumoniae*, conferred resistance to *S. pyogenes* isolates of *emm*-type 102.2, which lacked other aforementioned *dfr* genes. Trimethoprim may be more useful in treatment of *S. pyogenes* infections than previously thought. However, the herein described factors may lead to a rapid development and spread of resistance to this antibiotic agent in *S. pyogenes*. 
Introduction

Trimethoprim is used for the treatment of enteric, respiratory, skin, and urinary tract infections. It acts bacteriostatically by inhibition of the dihydrofolate reductase (DHFR), an enzyme of the folate synthesis pathway. Interference with this pathway inhibits bacterial DNA synthesis. Typically, trimethoprim is used in combination with sulfamethoxazole, a sulphonamide. This combination is also known as co-trimoxazole or SXT. Like other sulphonamides, sulfamethoxazole is an inhibitor of the dihydropteroate synthase, another enzyme of the folate synthesis pathway. Because of early non-standardized antibiotic susceptibility tests, *Streptococcus pyogenes* was considered as largely resistant to SXT. *S. pyogenes* is pathogenic in humans, causing a variety of diseases. This spectrum of diseases ranges from pharyngitis, tonsillitis and suppurative skin and soft tissue infections to severe invasive infections and immune sequelae.

Today’s knowledge about the pitfalls in SXT susceptibility testing raised doubts on some of the early data and the widespread resistance of *S. pyogenes* to this combination drug. SXT may be an underestimated alternative to other antibiotics under certain circumstances such as in the treatment of streptococcal skin and soft tissue co-infections with MRSA. However, a re-evaluation of SXT for use in *S. pyogenes* infections requires clinical studies and more, reliable data on the spread of resistance. The knowledge about the genes and mutations that confer resistance to *S. pyogenes* against sulphur antibiotics or trimethoprim is scant, but is a prerequisite for a comprehensive understanding of the extent of resistance and its development. Studies by Swedberg et. al. and Jönsson et. al. identified mutations in the chromosomally encoded dihydropteroate synthase as a cause for sulphonamide
resistance in *S. pyogenes*. In our previous work we report trimethoprim resistance in *S. pyogenes* due to a dihydrofolate reductase gene *dfr*G. To our knowledge, no other mutations and genes that confer resistance to trimethoprim or sulphonamides to *S. pyogenes* have been reported.

Generally, bacterial resistance to trimethoprim is mediated by the following five main mechanisms: (i) a permeability barrier, (ii) a naturally insensitive intrinsic DHFR, (iii) spontaneous mutations in the intrinsic DHFR, (iv) increased production of the sensitive target enzyme by up regulation of gene expression or gene duplication, and (v) horizontal acquisition (plasmid mediated or conjugation) of *dfr*-genes that encode resistant DHFRs. Only a few horizontally transmissible *dfr*-genes have been identified so far in Gram-positive bacteria (Tab. 1). Only one of them *dfr*G has been detected in *S. pyogenes*.

Although penicillin is in use against *S. pyogenes* for a long time, in vitro resistance against this antibiotic is not yet observed in this streptococcal species. However, treatment failure or adverse reactions to penicillin may occur. Therefore, development and spread of resistance to alternative antibiotics like erythromycin or clindamycin in *S. pyogenes* is a matter of concern. The use of SXT for treatment of skin infections has been suggested for certain settings. Trimethoprim is among the most frequently used antimicrobial agents and commonly prescribed in rural areas of India, from where high resistance rates to SXT have been reported. This required further examinations including investigations on the mechanisms that render *S. pyogenes* resistant to trimethoprim.
Materials and Methods

Bacterial strains

Indian S. pyogenes isolates were collected during a school survey and from clinical cases of human infections at the Postgraduate Institute of Medical Education and Research, Chandigarh (Northern India) and the Christian Medical College, Vellore (Southern India). S. pyogenes isolates from Germany were collected at the German National Reference Center for Streptococci. Details about the isolates from Germany and India are given in the supplemental table S1.

Antimicrobial susceptibility testing of S. pyogenes

Data from routine tests on SXT susceptibility of 2371 S. pyogenes isolates collected in Germany were analyzed at the German National Reference Center for Streptococci. The MICs for SXT were determined using the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (http://www.clsi.org). The microtiter plates (Sensititre NLMMCS10, TREK Diagnostic Systems Ltd., East Grinstead, UK) contained SXT (concentrations in mg/l: 0.25/4.75, 0.5/9.5, 1/19, 2/38, 4/76 and 8/152) with cation adjusted Mueller-Hinton broth (Oxoid, Wesel, Germany) and 5% lysed horse blood. The final inoculum was 5x10^5 CFU/ml. Incubation was carried out at 37°C for 24h in ambient air. S. pneumoniae ATCC 49619 was used as a control strain. Isolates with a MIC ≥2/38 mg/l of SXT were sent to the Helmholtz Centre for Infection Research (HZI).

Susceptibility to trimethoprim alone was tested at the HZI, using the following method of the European Committee on Antimicrobial Susceptibility Testing (EUCAST: www.eucast.org). Minimum inhibitory concentrations (MIC) of trimethoprim
were determined using the agar dilution method with a twofold dilution series from 512 mg/l to 1 mg/l of the antibiotic agent. Mueller-Hinton agar supplemented with 5% defibrinated horse blood and 20 mg/l ß-nicotinamide adenine dinucleotide was used. The MIC was determined as the lowest concentration of trimethoprim that inhibited visible growth after 18 hr of incubation at 37°C. *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains. Official breakpoints for resistance classification of *S. pyogenes* to trimethoprim were not available. Therefore, the isolates were classified tentatively as susceptible (MIC ≤ 2 mg/l), intermediate (MIC = 4 mg/l) or resistant (MIC > 4 mg/l) (see discussion). Our tentative classification was based on official breakpoints of the EUCAST for resistance of *S. pyogenes* to SXT (susceptible: ≤1/19 mg/l; resistant: >2/38 mg/l) and for *Streptococcus agalactiae* to trimethoprim alone (susceptible: ≤2 mg/l; resistant: >2 mg/l).

**DNA extraction and emm typing**

Genomic DNA was isolated with the Qiagen DNeasy kit (Qiagen, Hilden, Germany) after bacterial lysis with zirconia beads. The highly variable 5’-terminal nucleotide sequence of the *emm*-gene allows genotyping of *S. pyogenes* isolates. *Emm*-types were determined by amplification and sequencing of the 5’-end region of the *emm* gene using the primers *emm_fwd* and *emm_rev* (Tab. 2). The nucleotide sequences were compared against the *emm*-gene database of the Center for Disease Control and Prevention (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm).

**Standard PCR**

PCR was performed using 0.2 µM of suitable primers (Tab. 2), 0.2 mM of each dNTP, 1x Crimson Taq PCR Buffer, 1.5 mM MgCl₂, and 0.625 units of Crimson Taq
DNA polymerase (New England Biolabs) filled with water to a final volume of 25 µl.
The amplification conditions were: initial denaturation at 95°C for 1 min, 25 cycles of
denaturation at 95°C for 15 sec, annealing at gene-specific temperatures (Tab. 2) for
130 sec, and extension at 68°C for 1 min per kb of gene length. The cycle reaction
was followed by a final extension phase at 68°C for 5 min. PCR products were
analyzed by agarose gel electrophoresis.

**PCR detection of dfrG insertion sequence**

Isolates that were positive for dfrG were analyzed for the presence of the dfrG-
carrying insertion element with primers element_fwd and element_rev (Tab. 2)
specific for conserved regions that flank the integration site at genes that are
homologous to SPy_1769 of S. pyogenes SF370. This strain bore no integration
element (negative control) and therefore produced a 500 bp PCR product. Based on
our publication in 2012, a 3.8 kb amplification product was expected for dfrG-positive
isolates.

**Recombinant overexpression of the S. pyogenes intrinsic dihydrofolate
reductase gene dfr and determination of the MIC of overexpressing E. coli**

Intrinsic dfr genes of S. pyogenes isolates A981, A951, MGAS315 and SF370 were
recombinantly expressed in E. coli TOP10. To this end the dfr genes were amplified
using primers dfr_fwd and dfr_rev (Tab. 2). The amplification products were ligated
into the TA cloning vector pCR2.1 (Invitrogen). After transformation of E. coli TOP10
with the vector, the bacteria were grown on Luria-Bertani agar containing 100 mg/l
Ampicillin for selection of positive clones. Nucleotide sequences were verified by
DNA sequencing. The MIC of the E. coli clones was determined after cultivation on
Mueller-Hinton agar supplemented with trimethoprim in a twofold dilution series.
Nucleotide sequence of intrinsic \textit{dfr} genes and cluster analysis

Intrinsic \textit{dfr} genes were sequenced and novel sequence information was deposited in the GenBank database under accession numbers: 1359\_dfr (KF737388), A981\_dfr (KF737389), A1357\_dfr (KF737390), A842\_dfr (KF737391), A899\_dfr (KF737392). Cluster analysis was carried out with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using the neighbour joining algorithm.
Results

Antimicrobial susceptibility testing of isolates from India

A total of 268 *S. pyogenes* isolates that comprised 72 different *emm*-types was collected in India. The *emm*-type serves as a genotype marker for *S. pyogenes*. All isolates of the collection were examined for susceptibility to trimethoprim. In the agar dilution test 73 *S. pyogenes* isolates (27.2%) were intermediate (MIC 4 mg/l) or resistant to trimethoprim (MIC >4 mg/l). Of these 73 isolates, 65 resistant isolates showed MIC values above 16 mg/l. Four resistant isolates showed MIC values from 8 to 16 mg/l. The remaining 4 isolates that were classified as intermediate had MICs of 0 to 16 mg/l. The remaining 4 isolates that were classified as intermediate had MICs of 0 to 16 mg/l. Thus, 25.7% of the 268 *S. pyogenes* isolates from India were resistant to trimethoprim (n=69) and 1.5% intermediate (n=4).

Acquired trimethoprim resistance genes in *S. pyogenes*

Recently, we reported the presence of a trimethoprim resistance determinant *dfrG* in *S. pyogenes emm*1-2 from India. The *dfrG* gene was located chromosomally within an insertion sequence of about 3.3 kb. To determine the distribution of *dfrG* in *S. pyogenes* in India, all 268 isolates collected in India were tested by PCR. Out of these isolates 23 isolates (8.6%) were positive for *dfrG* (Fig. 1; Tab. 3), all of them resistant and highly tolerant to trimethoprim (MIC ≥256 mg/l). The previously characterized 3.3kb insertion element that contained *dfrG* was integrated into a gene that was homologous to SPy_1769 of *S. pyogenes* SF370 (integration site). As shown by PCR, the insertion element that contained *dfrG* was located in this integration site in 21 out of the 23 *dfrG*-positive isolates. The integration site of the two remaining isolates was not determined. As the majority of the intermediate or resistant isolates was negative for *dfrG* (50 out of 73), these isolates were examined...
by PCR for the presence of the trimethoprim resistance gene \textit{dfrF}. This PCR analysis identified \textit{dfrF} as a second acquired resistance gene in \textit{S. pyogenes}. The \textit{dfrF} gene was detected in 46 of the 268 isolates (17.2\%) of the Indian collection (Tab. 3; Fig. 2091). None of the isolates contained both \textit{dfrG} and \textit{dfrF}. Forty-two out of the 46 \textit{dfrF}-positive isolates were classified as resistant to trimethoprim by antimicrobial susceptibility testing with MICs equal to or higher than 32 mg/l. Three of the \textit{dfrF}-positive isolates were susceptible, one was intermediate. None of the known resistance genes \textit{dfrG}, \textit{dfrF}, \textit{dfrA}, \textit{dfrD} or \textit{dfrK} was detected in the seven remaining intermediate or resistant isolates. Four out of these seven isolates without known resistance factor belonged to \textit{emm}-type 102.2 and were classified as resistant (MIC 168-16 mg/l). One \textit{S. pyogenes} isolate of \textit{emm}-type 15.2 and two isolates of \textit{emm}-type 80.0 were intermediate (MIC 4 mg/l).

Trimethoprim resistance in SXT-resistant \textit{S. pyogenes} isolates from Germany

Resistance to SXT (MIC ≥2/38 mg/l) was observed for 37 out of 2371 isolates from Germany (1.6\%). These 37 isolates were tested for susceptibility to trimethoprim alone. Due to this pre-selection for SXT-resistance prior to trimethoprim susceptibility testing, a trimethoprim resistance rate for the complete collection of 2371 isolates from Germany could not be determined. Four out of the 37 SXT-resistant isolates were resistant to trimethoprim (MIC >512 mg/l) and positive for \textit{dfrF} (Tab. 4). Two of these isolates were of \textit{emm}-type 81, the other two of \textit{emm}-type 28 and \textit{stl854}, respectively. One trimethoprim resistant isolate with a MIC of 8 mg/l and twelve intermediate isolates (MIC 4 mg/l) were negative for \textit{dfrF} (Tab. 4). The resistance genes \textit{dfrG}, \textit{dfrA}, \textit{dfrK} or \textit{dfrD} were not detected in any of the 37 SXT-resistant
A point mutation in the intrinsic dihydrofolate reductase confers trimethoprim resistance to *S. pyogenes*

A total of 20 isolates from India and Germany, were intermediate or resistant to trimethoprim despite lacking an acquired *dfr* gene. Therefore, intrinsic *dfr* genes of representative isolates were examined by DNA sequencing for mutations and differences in the encoded DHFR. The cluster dendrogram based on the amino acid sequences of the intrinsic DHFRs (Fig. 2) includes sequences of *S. pyogenes* isolates for which whole genome sequences were available in the GenBank database. Despite belonging to different *emm*-types, 27 of the 59 analysed DHFR sequences were identical and formed one major cluster. The DHFRs of four type *emm*102.2 streptococci formed a distinct cluster. The respective isolates were all collected in India and resistant to trimethoprim (MIC 8 – 16 mg/l). As compared to DHFRs of susceptible and intermediate isolates, the DHFR of the resistant *emm*102.2 isolates harboured an amino acid substitution from isoleucine to leucine in position 100 (Fig. 3). The same substitution in the intrinsic DHFR was shown to be essential for trimethoprim resistance in *S. pneumoniae*.

To examine if the mutation in position 100 of the DHFR renders *S. pyogenes* resistant to trimethoprim, the intrinsic *dfr* genes of the resistant isolate A981 (*emm*102.2; MIC 16 mg/l), of the intermediate isolates A951 (*emm*80; MIC 4 mg/l) and MGAS315 (*emm*3; MIC 4 mg/l) and of the susceptible isolate SF370 (*emm*1; MIC 1 mg/l) were recombinantly expressed in *E. coli*. The *E. coli* clones were designated *E.c.-A981*, *E.c.-A951*, *E.c.-MGAS315* and *E.c.-SF370*, respectively.
DHFR of *S. pyogenes* SF370 differed from the DHFR of *S. pyogenes* MGAS315 and A951 in one amino acid, at position 19 in which DHFR of SF370 carried an isoleucine instead of a valine (Fig. 3). In the agar dilution test for trimethoprim resistance (Fig. 4) the wildtype *E. coli* was susceptible (MIC <1 mg/l). In accord with the trimethoprim susceptibility test of the *S. pyogenes* isolates, *E. coli* clone *E.c.*-SF370 that produced a DHFR with an isoleucine at position 19 was the most susceptible one. Higher tolerance was observed with the two clones *E.c.*-A951 and *E.c.*-MGAS315 that produced a DHFR with valine in position 19. The highest tolerance to trimethoprim was observed with clone *E.c.*-A981. This clone produced a DHFR with a valine in position 19. Moreover, in contrast to the other DHFRs, it harboured the substitution of isoleucine to leucine in position 100. Taken together the results indicate that a substitution of isoleucine by leucine in position 100 of the intrinsic DHFR caused trimethoprim resistance in *S. pyogenes*. 


Discussion

Commonly, trimethoprim is used together with sulfamethoxazole as a component of the combination drug SXT. Official breakpoints for classification of trimethoprim resistance of *S. pyogenes* are lacking. Therefore, we used tentative values (see materials and methods) that do not take into account clinical data and experiences on treatment of *S. pyogenes* infections with trimethoprim. Notably, out of the 215 isolates that were classified as susceptible in our study 128 and 87 had an MIC of ≤1 mg/l or ≤2 mg/l, respectively. Out of the 90 isolates that were not classified as susceptible, the considerable number of 16 isolates showed a lower tolerance (MIC 4 mg/l) as compared to the remaining 74 isolates (Tab. 3 and 4 and supplemental figure S2). Therefore, isolates with a MIC of 4 mg/l were classified as intermediate and isolates with higher MICs (≥8 mg/l) were classified as resistant.

Based on our classification, resistance to trimethoprim required the horizontal acquisition of *dfr*-genes or an isoleucine to leucine substitution in position 100 of the intrinsic DHFR. For one resistant isolate no resistance factor was identified, *S. pyogenes* A1308 from Germany (MIC 8 mg/l). Of the 74 resistant isolates from India and Germany 23 harboured *dfrG* (MIC 256 to >512 mg/l), 46 isolates harboured *dfrF* (MIC 32 to >512 mg/l) and the aforementioned substitution in the intrinsic DHFR was detected in four isolates (MIC 8 to 16 mg/l). To our knowledge this is the first description of *dfrF* and of the amino acid substitution in position 100 in the intrinsic DHFR as trimethoprim resistance factors in *S. pyogenes*. Notably, not all of the isolates that carried *dfrF* were resistant to trimethoprim. Three out of the 50 *dfrF*-positive isolates were susceptible to trimethoprim and one was intermediate, which could be due to low expression levels of this resistance gene. Still, *dfrF* was the most
frequent factor in India that conferred trimethoprim resistance to 15.6% of the *S. pyogenes* isolates. The *dfrF*-gene was present in isolates from Germany also, where it was the only detected trimethoprim resistance factor and caused resistance in 4 out of the 5 trimethoprim resistant isolates (Tab. 4). The high rate of 25.7% of trimethoprim resistant isolates was observed in India which may have been caused by frequent prescription of this drug in rural settings of India. When considered separately, the resistance rate was higher in Northern India (55.1%) than in Southern India (11.2%) (Tab. 3). A resistance rate for Germany was not determined, as the isolates of this study were pre-selected for SXT-resistance.

In a previous publication we described a 3.3 kb DNA sequence that contained *dfrG* and that was integrated into the genome of *S. pyogenes emm1-2* isolates. The element was integrated into a sequence that was homologous to SPy_1769. Recently, Bertsch et al. identified an identical 3.3 kb sequence, which was a part of the transposon Tn916 in *Listeria monocytogenes*. Other parts of the Tn916 transposon bore genetic elements for bacterial conjugation. Despite that a circular form of the 3.3 kb sequence was detected in *L. monocytogenes* the *dfrG* containing fragment could not be transferred on its own neither to *L. monocytogenes* nor to *Enterococcus faecalis*. Transfer of *dfrG* was only observed when it was part of Tn916, which provided genetic elements that were required for conjugation. Notably, we identified *S. pyogenes* isolates of 15 different *emm*-types that harboured the 3.3 kb *dfrG* containing sequence devoid of transposon sequences. In most of the isolates *dfrG* was integrated into the genome at the site mentioned above. The mode of transfer remains elusive.

In the present study, *dfrG* was more frequently detected in southern India as
compared to northern India. Two out of 179 isolates (1.1%) from southern India, both of \textit{emm}-type \textit{st}1732.1, harboured \textit{dfr}G. In contrast, 21 out of 89 isolates (23.6%) from northern India were positive for \textit{dfr}G. These 21 isolates belonged to 14 different \textit{emm}-types, which excludes that all isolates were clonal and suggests that horizontal transfer of \textit{dfr}G occurs with considerable frequency. Like \textit{dfr}G, \textit{dfr}F was found in isolates of various \textit{emm}-types. Conversely, \textit{dfr}F was more frequent in northern India where it was detected in 31 isolates (34.8%) of 14 different \textit{emm}-types. In southern India \textit{dfr}F was detected in 15 isolates (8.4%) of 5 different \textit{emm}-types. The nature of the DNA element that carries \textit{dfr}F remains unknown. To date, the resistance gene \textit{dfr}F has only been observed in different enterococcal species. Our data suggest that it is transferable to \textit{S. pyogenes} although the mode of transfer remains unknown. Taken together the herein presented data suggest a considerable horizontal transfer of \textit{dfr}G and \textit{dfr}F in \textit{S. pyogenes} in India.

As reported previously an amino acid substitution from isoleucine to leucine in position 100 of the intrinsic DHFR caused trimethoprim resistance in \textit{S. pneumoniae}. In our study the same mutation conferred trimethoprim resistance to all 4 \textit{S. pyogenes} isolates of \textit{emm}-type 102.2 that were isolated in India. The isolates differed in their MIC, which may be due to differences in the biosynthesis of the enzyme (Fig. 2). Taking previous observations into account, one can conclude that a single mutation either in the intrinsic dihydrofolate reductase gene or in the dihydropteroate synthase gene is sufficient to diminish the susceptibility of \textit{S. pyogenes} to trimethoprim or sulphonamides, respectively.

Recommendations against the use of SXT for \textit{S. pyogenes} infections continue in the belief that the pathogen is intrinsically resistant to this drug. As discussed recently by...
Bowen et. al. this seems to be a misconception. Studies reporting high resistance rates either used media known to have high concentrations of thymidine, which attenuate the antimicrobial effect of SXT, or did not provide details of the medium used. Today Mueller-Hinton medium is used in trimethoprim and SXT susceptibility testing to ensure a low thymidine concentration. As suggested by others SXT may be a valuable alternative for treatment of skin and soft tissue co-infections with S. pyogenes and methicillin resistant Staphylococcus aureus, in which treatment with penicillin is losing efficacy. The results of our study support the notion that the efficacy of trimethoprim for treatment of S. pyogenes infections in certain geographic regions is underestimated. However, we could identify factors that may readily cause resistance to trimethoprim in S. pyogenes and its rapid spread.
Acknowledgements

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References


detection of trimethoprim resistance determinant dfrG in Streptococcus
pyogenes clinical isolates in India. Antimicrob Agents Chemother 56:5424-
5425.

31:1451-1456.

amino acid mutation in the chromosome-encoded dihydrofolate reductase
confers trimethoprim resistance in Streptococcus pneumoniae. J Infect Dis
178:700-706.

structures of Escherichia coli and Lactobacillus casei dihydrofolate reductase
refined at 1.7 A resolution. I. General features and binding of methotrexate. J

14. Filman DJ, Bolin JT, Matthews DA, Kraut J. 1982. Crystal structures of
Escherichia coli and Lactobacillus casei dihydrofolate reductase refined at 1.7
A resolution. II. Environment of bound NADPH and implications for catalysis. J

15. Matthews DA, Bolin JT, Burridge JM, Filman DJ, Volz KW, Kaufman BT,
Beddell CR, Champness JN, Stammers DK, Kraut J. 1985. Refined crystal
structures of Escherichia coli and chicken liver dihydrofolate reductase

Dihydrofolate reductase. The stereochemistry of inhibitor selectivity. J Biol


Bapna JS, Tekur U, Gitanjali B, Shashindran CH, Pradhan SC,


Bertsch D, Uruty A, Anderegg J, Lacroix C, Perreten V, Meile L. 2013. Tn6198, a novel transposon containing the trimethoprim resistance gene dfrG.


Table 1: Horizontally transmissible dfr-genes in Gram-positive bacteria

<table>
<thead>
<tr>
<th>resistance gene</th>
<th>species</th>
<th>reference</th>
</tr>
</thead>
</table>
| dfrA            | Staphylococcus aureus  
                 | Staphylococcus epidermidis | Rouch et al.  
                 | Dale et al. |
| dfrD            | Staphylococcus haemolyticus  
                 | Listeria monocytogenes | Dale et al.  
                 | Charpentier & Courvalin |
| dfrK            | Staphylococcus pseudintermedius  
                 | Staphylococcus aureus  
                 | Staphylococcus hyicus  
                 | Enterococcus faecium  
                 | Enterococcus faecalis  
                 | Enterococcus durans/E. hirae  
                 | Enterococcus gallinarum/E. casseliflavus | Perreten et al.  
                 | Kadlec & Schwarz  
                 | Kadlec  
                 | Kadlec et al.  
                 | López et al.  
                 | López et al.  
                 | López et al. |
| dfrF            | different enterococcal species  
                 | Enterococcus faecalis | López et al.  
                 | Coque et al. |
| dfrG            | Enterococcus faecium  
                 | Staphylococcus aureus  
                 | Staphylococcus pseudintermedius | Listeria monocytogenes  
                 | Streptococcus pyogenes | Tanimoto & Ike.  
                 | Sekiguchi et al.  
                 | GenBank: FM004877.1  
                 | Bertsch et al.  
                 | Bergmann et al. |
Table 2: Oligonucleotides used in this study.

<table>
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<tr>
<th>name</th>
<th>sequence 5’-3’</th>
<th>amplicon size (bp)</th>
<th>annealing temperature (°C)</th>
<th>reference</th>
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<td>dfrG_fwd</td>
<td>ATGAAAGTTTCTTTGTAGCTGCGA CAATAAATTTTTCTCTCATACATG</td>
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<td>55</td>
<td>Bergmann et al.</td>
</tr>
<tr>
<td>dfrG_rev</td>
<td>TTACAGGGTCTGCGGCTATT TTCAAAGCCGTCAGTCAC</td>
<td>3800/500</td>
<td>55</td>
<td>Bergmann et al.</td>
</tr>
<tr>
<td>element_fwd</td>
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<td>52</td>
<td>Cattoir et al.</td>
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<tr>
<td>dfrA_fwd</td>
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<td>405</td>
<td>52</td>
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<td>dfrB_fwd</td>
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<td>52</td>
<td>Cattoir et al.</td>
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<td>52</td>
<td>Cattoir et al.</td>
</tr>
<tr>
<td>dfrD_fwd</td>
<td>GAGAATCCCAGAGGATTGG AAAATGTTTCTCATCAAA</td>
<td>422</td>
<td>55</td>
<td>López et al.</td>
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</tr>
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<td>dfrF_fwd</td>
<td>GTTCGTTTTTGGGACCTTTGAATCTTTAATCTCTTCGCTGACGCAATGCA</td>
<td>646</td>
<td>55</td>
<td>this study</td>
</tr>
<tr>
<td>dfrF_rev</td>
<td>GTTCGTTTTTGGGACCTTTGAATCTTTAATCTCTTCGCTGACGCAATGCA</td>
<td>646</td>
<td>55</td>
<td>this study</td>
</tr>
<tr>
<td>emm_fwd</td>
<td>TATTCGCTTAGAAAATTA AAATGTTTCTCATCAAA</td>
<td>1500</td>
<td>52</td>
<td>Facklam et al.</td>
</tr>
</tbody>
</table>
Table 3: Trimethoprim susceptibility and resistance factors in *S. pyogenes* isolates from India

<table>
<thead>
<tr>
<th>Region</th>
<th>No. of isolates</th>
<th>Detected resistance factor</th>
<th>Resistant (MIC &gt; 4 mg/l)</th>
<th>Intermediate (MIC = 4 mg/l)</th>
<th>Susceptible (MIC &lt; 4 mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>India</td>
<td>268 (100%)</td>
<td>dfrG 23 (8.6%) dfrF <em>46</em></td>
<td>69 (25.7%)</td>
<td>4 (1.5%)</td>
<td>195 (72.8%)</td>
</tr>
<tr>
<td>Northern</td>
<td>89 (100%)</td>
<td>dfrG 21 (23.6%) dfrF <em>31</em></td>
<td>49 (55.1%)</td>
<td>1 (1.1%)</td>
<td>39 (43.8%)</td>
</tr>
<tr>
<td>Southern</td>
<td>179 (100%)</td>
<td>dfrG 2 (1.1%) dfrF 15 (8.4%)</td>
<td>20 (11.2%)</td>
<td>3 (1.7%)</td>
<td>156 (87.1%)</td>
</tr>
</tbody>
</table>

* Other than for *dfrG* and the intrinsic resistant *dfr*, not all the isolates tested positive for *dfrF* were resistant to trimethoprim (see *2* and *3*)

*2* 42 out of 46 isolates were resistant; one isolate was intermediate; 3 isolates were susceptible

*3* 27 out of 31 isolates were resistant; one isolate was intermediate; three isolates were susceptible

*4* isolates were not tested for *dfrA*, *dfrD* and *dfrK*
Table 4: Trimethoprim susceptibility and trimethoprim resistance factors in SXT-resistant *S. pyogenes* isolates from Germany

<table>
<thead>
<tr>
<th>no. of isolates</th>
<th>detected resistance factor</th>
<th>resistant (MIC &gt; 4 mg/l)</th>
<th>intermediate (MIC = 4 mg/l)</th>
<th>susceptible (MIC &lt; 4 mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37 (100%)</td>
<td>dfrG</td>
<td>0</td>
<td>4 (10.8%)</td>
<td>12 (32.4%)</td>
</tr>
<tr>
<td></td>
<td>dfrF</td>
<td>5 *</td>
<td></td>
<td>20 (54.1%)</td>
</tr>
</tbody>
</table>

*1 all tested isolates were negative for *dfrA*, *dfrD* and *dfrK*

*2 4 out of 5 resistant isolates were positive for *dfrF*. In one isolate none of the known resistance factors was detected.
Figure 1: Distribution of the acquired resistant dihydrofolate reductase genes \textit{dfrG} and \textit{dfrF} and of an isoleucine to leucine substitution in position 100 of the intrinsic DHFR (I→L) in Indian \textit{S. pyogenes} isolates of different \textit{emm}-types (x-axis).
Figure 2: Cluster dendrogram of amino acid sequences of intrinsic dihydrofolate reductases of selected Indian and German S. pyogenes isolates and from S. pyogenes whole genome sequences, available at GenBank. Emm-types and MIC values (in mg/l) are indicated to the right of the isolate designation. DFHRs of emm102.2 isolates with an isoleucine to leucine substitution in position 100 of the amino acid sequence form a distinct clade that is highlighted in grey. Arrows indicate the DHRFs that are shown in Fig. 3 and those overexpressed in E. coli (Fig. 4.).
**Figure 3:** Comparison of amino acid sequences of the intrinsic DHFR of trimethoprim susceptible *E. coli* K-12, trimethoprim susceptible *S. pyogenes* SF370 and A1357, intermediate *S. pyogenes* MGAS315, A1374 and A951, and trimethoprim-resistant *S. pyogenes* A981 and *S. pneumoniae* AP13. Isolate designations, *emm*-types of *S. pyogenes* isolates and resistance classifications are given on the left. Indicated are amino acid positions involved in trimethoprim (●) and NADPH cofactor (□) binding of the *E. coli* K-12 enzyme. Differences between the *S. pyogenes* DHFRs are highlighted in gray. Identical amino acids (*), conserved substitutions (:), and semi-conserved substitutions (.) are indicated as determined by multiple alignment of all sequences. Amino acid positions are indicated on the right. A box indicates position 100.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Susceptibility</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> K-12</td>
<td>susceptible</td>
<td>...</td>
</tr>
<tr>
<td><em>S. pyogenes</em> SF370</td>
<td>susceptible</td>
<td>...</td>
</tr>
<tr>
<td><em>S. pyogenes</em> A1357</td>
<td>susceptible</td>
<td>...</td>
</tr>
<tr>
<td><em>S. pyogenes</em> A981</td>
<td>resistant</td>
<td>...</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> AP13</td>
<td>resistant</td>
<td>...</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>
Figure 4: Effect of trimethoprim on growth of *E. coli* TOP10 transformed with the intrinsic *dfr* genes of *S. pyogenes* strains SF370, MGAS315 and of the Indian *S. pyogenes* isolates A951 and A981 (Fig. 2). The *E. coli* clones are referred to as *E.c.*-SF370, *E.c.*-MGAS315, *E.c.*-A951 and *E.c.*-A981, respectively. The minimal inhibitory concentration was determined on Mueller-Hinton agar with a twofold dilution series of trimethoprim. The clone designation is shown on the right. Formation of a bright colony on the dark agar indicates growth in presence of trimethoprim in the concentration that is indicated above in mg/l. The figure shows one representative experiment out of three.