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# Factors That Cause Trimethoprim Resistance in *Streptococcus pyogenes*

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**The use of trimethoprim in treatment of *Streptococcus pyogenes* infections has long been discouraged because it has been 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 tested isolates from India and Germany and sought the factors that conferred the resistance. Resistant isolates were identified in tests for trimethoprim or trimethoprim-sulfamethoxazole (SXT) susceptibility. Resistant isolates were screened for the known horizontally transferable trimethoprim-insensitive dihydrofolate reductase (*dfr*) genes *dfrG*, *dfrF*, *dfrA*, *dfrD*, and *dfrK*. The nucleotide sequence of the intrinsic *dfr* gene 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%), *dfrF* appeared more frequently than *dfrG* (23 isolates; 33.3%) in India. The *dfrF* gene was also present in a collection of SXT-resistant isolates from Germany, in which it was the only detected trimethoprim resistance factor. The *dfrF* gene 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 factors described herein may lead to the rapid development and spread of resistance of *S. pyogenes* to this antibiotic agent.**

Trimethoprim is used for the treatment of enteric, respiratory, skin, and urinary tract infections (1). It acts bacteriostatically by inhibition of dihydrofolate reductase (DHFR), an enzyme of the folate synthesis pathway. Interference with this pathway inhibits bacterial DNA synthesis (2). Typically, trimethoprim is used in combination with sulfamethoxazole, a sulfonamide. This combination is also known as co-trimoxazole or SXT. Like other sulfonamides, sulfamethoxazole is an inhibitor of the dihydropteroate synthase, another enzyme of the folate synthesis pathway (3–5). Because of early nonstandardized antibiotic susceptibility tests, *Streptococcus pyogenes* has been considered 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 (6, 7).

Today's knowledge of the pitfalls in SXT susceptibility testing has raised doubts about some of the early data and the widespread resistance of *S. pyogenes* to this combination drug (8). SXT may be an underestimated alternative to other antibiotics under certain circumstances, such as in the treatment of streptococcal skin and soft tissue coinfections with methicillin-resistant *Staphylococcus aureus* (MRSA) (8, 9). However, a reevaluation of SXT for use in *S. pyogenes* infections requires clinical studies and more, reliable data on the spread of resistance. Knowledge about the genes and mutations that confer resistance to *S. pyogenes* against sulfur 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. (4) and Jönsson et al. (3) identified mutations in the chromosomally encoded dihydropteroate synthase as a cause for sulfonamide resistance in *S. pyogenes*. In our previous work, we report trimethoprim resistance in *S. pyogenes* due to the dihydrofolate reductase (*dfr*) gene *dfrG* (10). To our knowledge, no other mutations and genes that confer resistance to trimethoprim or sulfonamides to *S. pyogenes* have been reported.

Generally, bacterial resistance to trimethoprim is mediated by the following five main mechanisms: (i) a permeability barrier (1, 11), (ii) a naturally insensitive intrinsic DHFR (1), (iii) spontaneous mutations in the intrinsic DHFR (12–17), (iv) increased production of the sensitive target enzyme by upregulation of gene expression or gene duplication (18), 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 (Table 1). Only one of them, the *dfrG* gene, has been detected in *S. pyogenes*.

Although penicillin has been used against *S. pyogenes* for a long time, *in vitro* resistance against this antibiotic has not yet been observed for this streptococcal species. However, treatment failure (19, 20) or adverse reactions to penicillin (21) may occur. Therefore, the development and spread of resistance of *S. pyogenes* to alternative antibiotics, such as erythromycin or clindamycin, is a matter of concern. The use of SXT for treatment of skin infections has been suggested for use in certain settings (8). Trimethoprim is among the most frequently used antimicrobial agents (22, 23) and is commonly prescribed in rural areas of India

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**TABLE 1** Horizontally transmissible *dfr* genes in Gram-positive bacteria

| Resistance gene | Species | Reference or GenBank accession no. |
|---|---|---|
| *dfrA* | *Staphylococcus aureus* | 33 |
| | *Staphylococcus epidermidis* | 34 |
| *dfrD* | *Staphylococcus haemolyticus* | 34 |
| | *Listeria monocytogenes* | 35 |
| *dfrK* | *Staphylococcus pseudintermedius* | 36 |
| | *Staphylococcus aureus* | 37 |
| | *Staphylococcus hyicus* | 38 |
| | *Enterococcus faecium* | 38 |
| | *Enterococcus faecalis* | 32 |
| | *Enterococcus durans*, *Enterococcus hirae* | 32 |
| | *Enterococcus gallinarum*, *Enterococcus casseliflavus* | 32 |
| *dfrF* | Various enterococcal species | 32 |
| | *Enterococcus faecalis* | 31 |
| *dfrG* | *Enterococcus faecium* | 39 |
| | *Staphylococcus aureus* | 40 |
| | *Staphylococcus pseudintermedius* | GenBank accession no. FM204877.1 |
| | *Listeria monocytogenes* | 30 |
| | *Streptococcus pyogenes* | 10 |

(24), where high resistance rates to SXT have been reported (25, 26). This required further examination, including investigations of 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 infection at the Postgraduate Institute of Medical Education and Research, Chandigarh (northern India), and the Christian Medical College, Vellore (southern India) (27, 28). *S. pyogenes* isolates from Germany were collected at the German National Reference Center for Streptococci. For details about the isolates from Germany and India, see Table S1 in the supplemental material.

**Antimicrobial susceptibility testing of *S. pyogenes*.** Data from routine tests of SXT susceptibility of 2,371 *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, United Kingdom) contained trimethoprim-sulfamethoxazole (SXT) in the ratio 1:19 (the concentrations [trimethoprim/sulfamethoxazole], in mg/liter, were 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 $5 \times 10^5$ CFU/ml. Incubation was carried out at 37°C for 24 h in ambient air. *Streptococcus pneumoniae* ATCC 49619 was used as a control strain. Isolates with a MIC of ≥2/38 mg/liter of SXT were sent to the Helmholtz Centre for Infection Research (HZI).

Susceptibility to trimethoprim alone was tested at the HZI by using the following method of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org). MICs of trimethoprim were determined using the agar dilution method with a 2-fold dilution series from 512 mg/liter to 1 mg/liter of the antibiotic agent. Mueller-Hinton agar supplemented with 5% defibrinated horse blood and 20 mg/liter β-NAD was used. The MIC was determined as the lowest concentration of trimethoprim that inhibited visible growth after 18 h 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/liter), intermediate (MIC, 4 mg/liter), or resistant (MIC, >4 mg/liter) (see Discussion). Our tentative classification was based on official breakpoints of the EUCAST for resistance of *S. pyogenes* to SXT (susceptible, ≤1/19 mg/liter; resistant, >2/38 mg/liter) and for *Streptococcus agalactiae* to trimethoprim alone (susceptible, ≤2 mg/liter; resistant, >2 mg/liter).

**DNA extraction and *emm* typing.** Genomic DNA was isolated with a 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 (forward) and emm_rev (reverse) (Table 2). The nucleotide sequences were compared against the *emm* gene

**TABLE 2** Oligonucleotides used in this study

| Name | Sequence (5′ to 3′) | Amplicon size (bp) | Annealing temp (°C) | Reference or source |
|---|---|---|---|---|
| dfrG_fwd | ATGAAAGTTTCTTTGATTGCTGCGA | 500 | 55 | 10 |
| dfrG_rev | CAATAAGTTTTTTCTTTCATATACATG | | | |
| element_fwd | TTACAGGGTCTGCGGCTATT | 3,800/500[a] | 55 | 10 |
| element_rev | TTCAAAGCCGTCTCAGTCAC | | | |
| dfrA_fwd | ATCAATAATTGTCGCTCACG | 405 | 52 | 41 |
| dfrA_rev | ACTGAAGATTCGACTTCCC | | | |
| dfrD_fwd | GCAAGGATAACGACATTCC | 202 | 52 | 41 |
| dfrD_rev | GCAGCTTCTATTGAATGGG | | | |
| dfrF_fwd | TTAACAACGGGTAATGTGGT | 201 | 52 | 41 |
| dfrF_rev | AAATAGTCCATATCCACCAG | | | |
| dfrK_fwd | GAGAATCCCAGAGGATTGGG | 422 | 55 | 32 |
| dfrK_rev | CAAGAAGCTTTTCGCTCATAAA | | | |
| dfr_fwd | GTTGCACTTTGACCTTGCTATTTAA | 646 | 55 | This study |
| dfr_rev | ATACTCCAAAAATACTACGTTGCAT | | | |
| emm_fwd | TATTCGCTTAGAAAATTAA | 1,500 | 52 | 29 |
| emm_rev | GCAAGTTCTTCAGCTTGTTT | | | |

[a] The two amplicon sizes refer to amplicons with and without *dfrG*-carrying sequences, respectively.

**TABLE 3** Trimethoprim susceptibility and resistance factors in *S. pyogenes* isolates from India

| Region | No. (%) of isolates | | | | | |
|---|---|---|---|---|---|---|
| | Total | Resistance factor detected[d] | | Resistant (MIC > 4 mg/liter) | Intermediate (MIC = 4 mg/liter) | Susceptible (MIC < 4 mg/liter) |
| | | *dfrG* | *dfrF*[a] | | | |
| India | 268 (100) | 23 (8.6) | 46[b] (17.2) | 69 (25.7) | 4 (1.5) | 195 (72.8) |
| Northern India | 89 (100) | 21 (23.6) | 31[c] (34.8) | 49 (55.1) | 1 (1.1) | 39 (43.8) |
| Southern India | 179 (100) | 2 (1.1) | 15 (8.4) | 20 (11.2) | 3 (1.7) | 156 (87.1) |

[a] Not all isolates that tested positive for the *dfrF* resistance gene were resistant to trimethoprim (see footnotes *b* and *c*).
[b] Forty-two out of 46 isolates were resistant, 1 isolate was intermediate, and 3 isolates were susceptible.
[c] Twenty-seven out of 31 isolates were resistant, 1 isolate was intermediate, and 3 isolates were susceptible.
[d] Isolates were not tested for the resistance genes *dfrA*, *dfrD*, and *dfrK*.

database of the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm).

**Standard PCR.** PCR was performed using 0.2 μM suitable primers (Table 2), 0.2 mM (each) dNTP, 1× Crimson *Taq* PCR buffer, 1.5 mM $MgCl_2$, and 0.625 units of Crimson *Taq* DNA polymerase (New England BioLabs), with the addition of water to a final volume of 25 μl. The amplification conditions were as follows: initial denaturation at 95°C for 1 min, 25 cycles of denaturation at 95°C for 15 s, annealing at gene-specific temperatures (Table 2) for 30 s, 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 the *dfrG* insertion sequence.** Isolates that were positive for the *dfrG* resistance gene were analyzed for the presence of the *dfrG* gene-carrying insertion element with primers element_fwd and element_rev (Table 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 2012 publication (10), 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 *Escherichia coli* TOP10. To this end, the *dfr* genes were amplified using primers dfr_fwd and dfr_rev (Table 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/liter ampicillin for selection of positive clones. Nucleotide sequences were verified by DNA sequencing. The MICs of the *E. coli* clones were determined after cultivation on Mueller-Hinton agar supplemented with trimethoprim in a 2-fold dilution series.

**Nucleotide sequence of intrinsic *dfr* genes and cluster analysis.** Intrinsic *dfr* genes were sequenced and novel sequence information was deposited in the GenBank database under the following accession numbers: A1359_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 neighbor 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 (27, 28). The *emm* type serves as a genotype marker for *S. pyogenes* (29). All isolates in the collection were examined for susceptibility to trimethoprim. In the agar dilution test, 73 *S. pyogenes* isolates (27.2%) were intermediate (MIC, 4 mg/liter) or resistant (MIC, >4 mg/liter) to trimethoprim. Of these 73 isolates, 65 resistant isolates showed MIC values above 16 mg/liter. Four resistant isolates showed MIC values of 8 to 16 mg/liter. The remaining 4 isolates that were classified as intermediate had MICs of 4 mg/liter (Table 3) (see Table S1 in the supplemental material). 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, the *dfrG* gene, in *S. pyogenes emm* type 1-2 (*emm*1-2) from India. The *dfrG* gene was located chromosomally within an insertion sequence of about 3.3 kb (10). To determine the distribution of the *dfrG* gene in *S. pyogenes* in India, all 268 isolates collected in India were tested by PCR. Of these isolates, 23 (8.6%) were positive for *dfrG* (Fig. 1; Table 3), all of them resistant and highly tolerant to trimethoprim (MIC, ≥256 mg/liter). The previously characterized 3.3-kb insertion element that contained the *dfrG* gene 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 the *dfrG* gene 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 *dfrF*. This PCR analysis identified *dfrF* as a second acquired resistance gene in *S. pyogenes*. The *dfrF* gene was detected in 46 of the 268 isolates (17.2%) of the Indian collection (Table 3; Fig. 1). None of the isolates contained both *dfrG* and *dfrF* genes. Forty-two out of the 46 *dfrF*-positive isolates were classified as resistant to trimethoprim by antimicrobial susceptibility testing, with MICs equal to or higher than 32 mg/liter. Three of the *dfrF*-positive isolates were susceptible, and one was intermediate. None of the known trimethoprim resistance genes (*dfrG*, *dfrF*, *dfrA*, *dfrD*, and *dfrK*) was detected in the seven remaining intermediate or resistant isolates. Four out of these seven isolates without a known resistance factor belonged to *emm* type 102.2 and were classified as resistant (MIC range, 8 to 16 mg/liter). One *S. pyogenes* isolate of *emm* type 15.2 and two isolates of *emm* type 80.0 were intermediate (MIC, 4 mg/liter).

**Trimethoprim resistance in SXT-resistant *S. pyogenes* isolates from Germany.** Resistance to SXT was observed for 37 out of 2,371 isolates from Germany (1.6%). These 37 isolates were tested for susceptibility to trimethoprim alone. Due to this preselection for SXT resistance prior to trimethoprim susceptibility testing, a trimethoprim resistance rate for the complete collection of 2,371 isolates from Germany could not be determined. Four out of the 37 SXT-resistant isolates were resistant to trimethoprim (MIC,

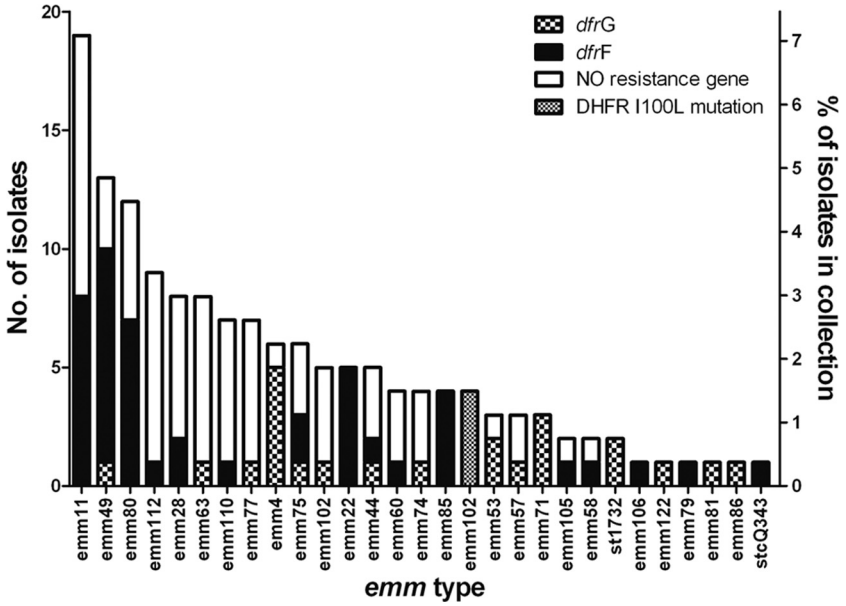


**FIG 1** Distribution of the acquired resistant dihydrofolate reductase genes *dfrG* and *dfrF* and of an isoleucine-to-leucine substitution at position 100 of the intrinsic DHFR (I→L) in Indian *S. pyogenes* isolates of different *emm* types (*x* axis).

>512 mg/liter) and positive for the resistance gene *dfrF* (Table 4). Two of these isolates were of *emm* type 81, and the others were of *emm* type 28 and *st* 854, respectively. One trimethoprim-resistant isolate with a MIC of 8 mg/liter and 12 intermediate isolates (MIC, 4 mg/liter) were negative for *dfrF* (Table 4). The resistance genes *dfrG*, *dfrA*, *dfrK*, and *dfrD* were not detected in any of the 37 SXT-resistant isolates from Germany.

**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 analyzed DHFR sequences were identical and formed one major cluster. The DHFRs of four *emm*102.2 streptococci formed a distinct cluster. The respective isolates were all collected in India and resistant to trimethoprim (MICs, 8 to 16 mg/liter). Compared to DHFRs of susceptible and intermediate isolates, the DHFR of the resistant *emm*102.2 isolates harbored an amino acid substitution of leucine for the isoleucine at position 100 (Fig. 3). The same substitution in the intrinsic DHFR was shown to be essential for trimethoprim resistance in *S. pneumoniae* (12, 17).

To examine if the mutation at 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/liter), of the intermediate isolates A951 (*emm*80; MIC, 4 mg/liter) and MGAS315 (*emm*3; MIC, 4 mg/liter), and of the susceptible isolate SF370 (*emm*1; MIC, 1 mg/liter) 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. The DHFR of *S. pyogenes* SF370 differed from the DHFR of *S. pyogenes* MGAS315 and A951 by one amino acid, at position 19, at which DHFR of SF370 carried an isoleucine instead of a valine (Fig. 3). In the agar dilution test for trimethoprim resistance (Fig. 4), the wild-type *E. coli* isolate was susceptible (MIC, <1 mg/liter). In accord with the trimethoprim susceptibility test of the *S. pyogenes* isolates, *E. coli* clone *E.c.*-SF370, which 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, which produced a DHFR with valine at position 19. The highest tolerance to trimethoprim was observed with clone *E.c.*-A981. This clone produced a DHFR with a valine at position 19. Moreover, in contrast to the other DHFRs, it harbored the replacement of isoleucine with leucine at position 100. Taken together, the results indicate that a replacement of isoleucine with leucine at position 100 of the intrinsic DHFR caused trimethoprim resistance in *S. pyogenes*.

**TABLE 4** Trimethoprim susceptibility and trimethoprim resistance factors in SXT-resistant *S. pyogenes* isolates from Germany

| No. (%) of isolates | | | | | |
|---|---|---|---|---|---|
| | Resistance factor detected[a] | | Resistant (MIC > 4 mg/liter) | Intermediate (MIC = 4 mg/liter) | Susceptible (MIC < 4 mg/liter) |
| Total | *dfrG* | *dfrF* | | | |
| 37 (100) | 0 | 4 (10.8) | 5[b] (13.5) | 12 (32.4) | 20 (54.1) |

[a] All isolates tested were negative for the resistance genes *dfrA*, *dfrD*, and *dfrK*.
[b] Four out of 5 resistant isolates were positive for the *dfrF* resistance gene. For one isolate, none of the known resistance factors was detected.

## DISCUSSION

Commonly, trimethoprim is used together with sulfamethoxazole as a component of the combination drug SXT. Official breakpoints for the 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

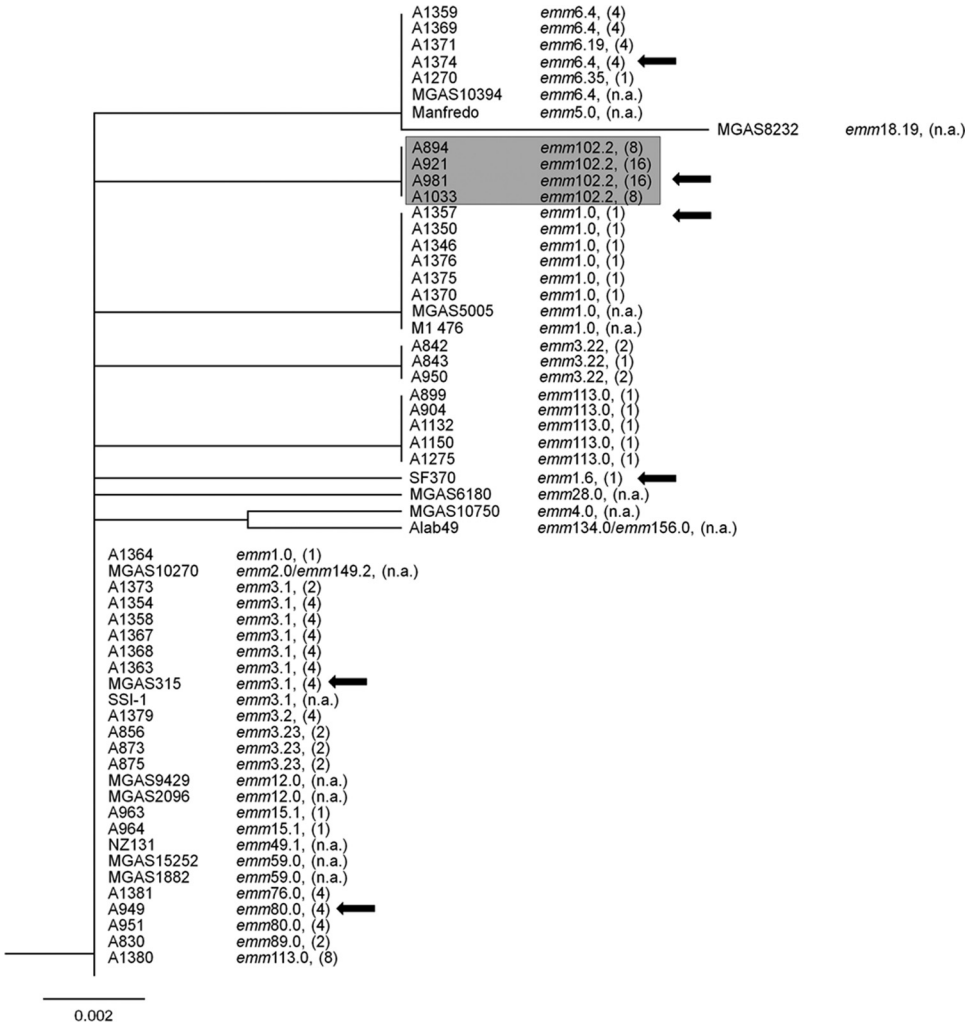


**FIG 2** Cluster dendrogram of amino acid sequences of intrinsic dihydrofolate reductases of selected Indian and German *S. pyogenes* isolates and from *S. pyogenes* whole-genome sequences, available in GenBank. *emm* types and MIC values (in mg/liter) are indicated to the right of the isolate designation. DFHRs of *emm*102.2 isolates with an isoleucine-to-leucine substitution at position 100 of the amino acid sequence form a distinct clade that is highlighted in gray. Arrows indicate the DHRFs that are shown in Fig. 3 and those overexpressed in *E. coli* (Fig. 4.). n.a., not available.

experiences with the 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 a MIC of ≤1 mg/liter and ≤2 mg/liter, respectively. Out of the 90 isolates that were not classified as susceptible, a considerable number, 16 isolates, showed a lower tolerance (MIC, 4 mg/liter) than that of the remaining 74 isolates (Tables 3 and 4) (see Fig. S2 in the supplemental material). Therefore, isolates with a MIC of 4 mg/liter were classified as intermediate and isolates with higher MICs (≥8 mg/liter) were classified as resistant.

Based on our classification, resistance to trimethoprim required the horizontal acquisition of *dfr* genes or an isoleucine-to-leucine substitution at position 100 of the intrinsic DHFR. For one resistant isolate, *S. pyogenes* A1308 from Germany (MIC, 8 mg/liter), no resistance factor was identified. Of the 74 resistant isolates from India and Germany, 23 harbored the *dfrG* resistance gene (MICs, 256 to >512 mg/liter), 46 isolates harbored the *dfrF* resistance gene (MICs, 32 to >512 mg/liter), and the aforementioned substitution in the intrinsic DHFR was detected in four isolates (MICs, 8 to 16 mg/liter). To our knowledge, this is the first description of the *dfrF* gene and of the amino acid substitution at position 100 in the intrinsic DHFR as trimethoprim resistance factors in *S. pyogenes*. Notably, not all of the isolates that carried the *dfrF* gene 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 the *S. pyogenes* isolates and was present in 15.6% of the isolates. The *dfrF* gene was present in isolates from Germany also, where it was the only trimethoprim resistance factor detected and caused resistance in 4 out of the 5 trimethoprim-resistant isolates (Table 4). The high rate of 25.7% isolates with trimethoprim resistance was observed in India; this may have been caused by frequent prescription of this drug in rural settings of India (24, 25). When considered separately, the resistance rate was higher in northern India (55.1%) than in southern India (11.2%) (Table 3). A resistance


**FIG 3** Comparison of amino acid sequences of the intrinsic DHFR of trimethoprim-susceptible *E. coli* K-12, trimethoprim-susceptible *S. pyogenes* SF370 and A1357, trimethoprim-intermediate *S. pyogenes* MGAS315, A1374, and A951, and trimethoprim-resistant *S. pyogenes* A981 and *S. pneumoniae* AP13 (12). 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 (13–16). Differences between the *S. pyogenes* DHFRs are highlighted in gray. Identical amino acids (*), conserved substitutions (:), and semiconserved substitutions (.) are indicated as determined by multiple alignment of all sequences. Amino acid positions are indicated on the right. A box indicates position 100.

rate for Germany was not determined, as the isolates of this study were preselected for SXT resistance.

In a previous publication (10), we described a 3.3-kb DNA sequence that contained the *dfrG* gene and that was integrated into the genome of *S. pyogenes emm*1-2 isolates. The element was integrated into a sequence that was homologous to SPy_1769. Recently, Bertsch et al. (30) identified an identical 3.3-kb sequence, which was a part of the transposon Tn*916* in *Listeria monocytogenes*. Other parts of the Tn*916* transposon bore genetic elements for bacterial conjugation. Although 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 to *L. monocytogenes* or to *Enterococcus faecalis* (30). Transfer of the *dfrG* gene was observed only when it was part of Tn*916*, which provided genetic elements that were required for conjugation. Notably, we identified *S. pyogenes* isolates of 15 different *emm* types that harbored the 3.3-kb *dfrG*-containing sequence devoid of transposon sequences. In most of the isolates, the *dfrG* gene was integrated into the genome at the site mentioned above. The mode of transfer remains elusive.

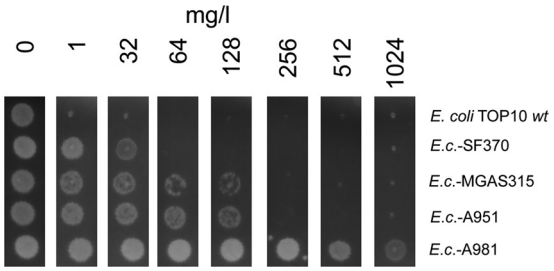


**FIG 4** Effect of trimethoprim on growth of *E. coli* TOP10 transformed with the intrinsic *dfr* genes of *S. pyogenes* strains SF370 and MGAS315 and of the Indian *S. pyogenes* isolates A951 and A981 (Fig. 2). The *E. coli* clones are referred to as *E.c.*-SF370, *E.c.*-MGAS315, *E.c.*-A951, and *E.c.*-A981, respectively. The MIC was determined on Mueller-Hinton agar with a 2-fold dilution series of trimethoprim. The clone designation is shown on the right. Formation of a bright colony on the dark agar indicates growth in the presence of trimethoprim in the concentration that is indicated above in mg/liter. The figure shows one representative experiment out of three.

In the present study, the *dfrG* gene was more frequently detected in southern India than in northern India. Two out of 179 isolates (1.1%) from southern India, both of *emm* type *st*1732.1, harbored *dfrG*. In contrast, 21 out of 89 isolates (23.6%) from northern India were positive for *dfrG*. These 21 isolates belonged to 14 different *emm* types, which excludes the possibility that all isolates were clonal and suggests that horizontal transfer of the *dfrG* gene occurs with considerable frequency. Like the *dfrG* gene, the *dfrF* gene was found in isolates of various *emm* types. Conversely, *dfrF* was more frequent in northern India, where it was detected in 31 isolates (34.8%) of 14 different *emm* types. In southern India, *dfrF* was detected in 15 isolates (8.4%) of 5 different *emm* types. The nature of the DNA element that carries the *dfrF* gene remains unknown. To date, the resistance gene *dfrF* has only been observed in different enterococcal species (31, 32). Our data suggest that it is transferable to *S. pyogenes*, although the mode of transfer remains unknown. Taken together, the data presented herein suggest a considerable horizontal transfer of *dfrG* and *dfrF* in *S. pyogenes* in India.

As reported previously, an amino acid substitution of isoleucine with leucine at position 100 of the intrinsic DHFR caused trimethoprim resistance in *S. pneumoniae* (12, 17). In our study, the same mutation conferred trimethoprim resistance to all 4 *S. pyogenes* isolates of *emm* type 102.2 that were isolated in India. The isolates differed in their MICs, which may be due to differences in 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 (3, 4) is sufficient to diminish the susceptibility of *S. pyogenes* to trimethoprim or sulfonamides, respectively.

Recommendations against the use of SXT for *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 attenuates 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 (8). As suggested by others, SXT may be a valuable alternative for treatment of skin and soft tissue coinfections with *S. pyogenes* and methicillin-resistant *Staphylococcus aureus*, for which penicillin treatment is losing efficacy (8). 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 also identified factors that may readily cause resistance to trimethoprim in *S. pyogenes* and its rapid spread.

## REFERENCES


1. **Huovinen P, Sundstrom L, Swedberg G, Skold O.** 1995. Trimethoprim and sulfonamide resistance. Antimicrob. Agents Chemother. **39:**279–289. http://dx.doi.org/10.1128/AAC.39.2.279.
2. **Miovic M, Pizer LI.** 1971. Effect of trimethoprim on macromolecular synthesis in Escherichia coli. J. Bacteriol. **106:**856–862.
3. **Jönsson M, Strom K, Swedberg G.** 2003. Mutations and horizontal transmission have contributed to sulfonamide resistance in Streptococcus pyogenes. Microb. Drug Resist. **9:**147–153. http://dx.doi.org/10.1089/107662903765826732.
4. **Swedberg G, Ringertz S, Skold O.** 1998. Sulfonamide resistance in Streptococcus pyogenes is associated with differences in the amino acid sequence of its chromosomal dihydropteroate synthase. Antimicrob. Agents Chemother. **42:**1062–1067.
5. **Bushby SR.** 1975. Synergy of trimethoprim-sulfamethoxazole. Can. Med. Assoc. J. **112:**63–66.
6. **Chhatwal GS, Graham RM.** 2008. Streptococcal diseases, p 231–241. *In* Encyclopedia of public health 2008. Academic Press, San Diego, CA.
7. **Nitsche-Schmitz DP, Chhatwal GS.** 2013. Host-pathogen interactions in streptococcal immune sequelae. Curr. Top. Microbiol. Immunol. **368:**155–171. http://dx.doi.org/10.1007/82_2012_296.
8. **Bowen AC, Lilliebridge RA, Tong SY, Baird RW, Ward P, McDonald MI, Currie BJ, Carapetis JR.** 2012. Is Streptococcus pyogenes resistant or susceptible to trimethoprim-sulfamethoxazole? J. Clin. Microbiol. **50:**4067–4072. http://dx.doi.org/10.1128/JCM.02195-12.
9. **Tong SY, Andrews RM, Kearns T, Gundjirryirr R, McDonald MI, Currie BJ, Carapetis JR.** 2010. Trimethoprim-sulfamethoxazole compared with benzathine penicillin for treatment of impetigo in Aboriginal children: a pilot randomised controlled trial. J. Paediatr. Child Health **46:**131–133. http://dx.doi.org/10.1111/j.1440-1754.2010.01697.x.
10. **Bergmann R, Sagar V, Nitsche-Schmitz DP, Chhatwal GS.** 2012. First detection of trimethoprim resistance determinant dfrG in Streptococcus pyogenes clinical isolates in India. Antimicrob. Agents Chemother. **56:**5424–5425. http://dx.doi.org/10.1128/AAC.01284-12.
11. **Huovinen P.** 1987. Trimethoprim resistance. Antimicrob. Agents Chemother. **31:**1451–1456. http://dx.doi.org/10.1128/AAC.31.10.1451.
12. **Pikis A, Donkersloot JA, Rodriguez WJ, Keith JM.** 1998. A conservative amino acid mutation in the chromosome-encoded dihydrofolate reductase confers trimethoprim resistance in Streptococcus pneumoniae. J. Infect. Dis. **178:**700–706. http://dx.doi.org/10.1086/515371.
13. **Bolin JT, Filman DJ, Matthews DA, Hamlin RC, Kraut J.** 1982. Crystal structures of Escherichia coli and Lactobacillus casei dihydrofolate reductase refined at 1.7 Å resolution. I. General features and binding of methotrexate. J. Biol. Chem. **257:**13650–13662.
14. **Filman DJ, Bolin JT, Matthews DA, Kraut J.** 1982. Crystal structures of Escherichia coli and Lactobacillus casei dihydrofolate reductase refined at 1.7 Å resolution. II. Environment of bound NADPH and implications for catalysis. J. Biol. Chem. **257:**13663–13672.
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 containing bound trimethoprim. J. Biol. Chem. **260:**381–391.
16. **Matthews DA, Bolin JT, Burridge JM, Filman DJ, Volz KW, Kraut J.** 1985. Dihydrofolate reductase. The stereochemistry of inhibitor selectivity. J. Biol. Chem. **260:**392–399.
17. **Adrian PV, Klugman KP.** 1997. Mutations in the dihydrofolate reductase gene of trimethoprim-resistant isolates of Streptococcus pneumoniae. Antimicrob. Agents Chemother. **41:**2406–2413.
18. **Brochet M, Couve E, Zouine M, Poyart C, Glaser P.** 2008. A naturally occurring gene amplification leading to sulfonamide and trimethoprim resistance in Streptococcus agalactiae. J. Bacteriol. **190:**672–680. http://dx.doi.org/10.1128/JB.01357-07.
19. **Brook I.** 2001. Failure of penicillin to eradicate group A beta-hemolytic streptococci tonsillitis: causes and management. J. Otolaryngol. **30:**324–329. http://dx.doi.org/10.2310/7070.2001.19359.
20. **Kaplan EL, Johnson DR.** 2001. Unexplained reduced microbiological efficacy of intramuscular benzathine penicillin G and of oral penicillin V in eradication of group a streptococci from children with acute pharyngitis. Pediatrics **108:**1180–1186. http://dx.doi.org/10.1542/peds.108.5.1180.
21. **Macy E, Ngor E.** 4 April 2013. Recommendations for the management of beta-lactam intolerance. Clin. Rev. Allergy Immunol. http://dx.doi.org/10.1007/s12016-013-8369-8.
22. **Coenen S, Adriaenssens N, Versporten A, Muller A, Minalu G, Faes C, Vankerckhoven V, Aerts M, Hens N, Molenberghs G, Goossens H, ESAC Project Group.** 2011. European Surveillance of Antimicrobial Consumption (ESAC): outpatient use of tetracyclines, sulphonamides and trimethoprim, and other antibacterials in Europe (1997-2009). J. Antimicrob. Chemother. **66**(Suppl 6):vi57–vi70. http://dx.doi.org/10.1093/jac/dkr458.
23. **McClean P, Hughes C, Tunney M, Goossens H, Jans B, European Surveillance of Antimicrobial Consumption Nursing Home Project G.** 2011. Antimicrobial prescribing in European nursing homes. J. Antimicrob. Chemother. **66:**1609–1616. http://dx.doi.org/10.1093/jac/dkr183.
24. **Bapna JS, Tekur U, Gitanjali B, Shashindran CH, Pradhan SC, Thulasimani M, Tomson G.** 1992. Drug utilization at primary health care level in southern India. Eur. J. Clin. Pharmacol. **43:**413–415. http://dx.doi.org/10.1007/BF02220618.
25. **Devi U, Borah PK, Mahanta J.** 2011. The prevalence and antimicrobial susceptibility patterns of beta-hemolytic streptococci colonizing the throats of schoolchildren in Assam, India. J. Infect. Dev. Ctries. **5:**804–808. http://dx.doi.org/10.3855/jidc.1465.
26. **Jain A, Shukla VK, Tiwari V, Kumar R.** 2008. Antibiotic resistance pattern of group-A beta-hemolytic streptococci isolated from north Indian children. Indian J. Med. Sci. **62:**392–396. http://dx.doi.org/10.4103/0019-5359.44018.
27. **Sagar V, Bergmann R, Nerlich A, McMillan DJ, Nitsche Schmitz DP, Chhatwal GS.** 2012. Variability in the distribution of genes encoding virulence factors and putative extracellular proteins of Streptococcus pyogenes in India, a region with high streptococcal disease burden, and implication for development of a regional multisubunit vaccine. Clin. Vaccine Immunol. **19:**1818–1825. http://dx.doi.org/10.1128/CVI.00112-12.
28. **Haggar A, Nerlich A, Kumar R, Abraham VJ, Brahmadathan KN, Ray P, Dhanda V, Joshua JM, Mehra N, Bergmann R, Chhatwal GS, Norrby-Teglund A.** 2012. Clinical and microbiologic characteristics of invasive Streptococcus pyogenes infections in north and south India. J. Clin. Microbiol. **50:**1626–1631. http://dx.doi.org/10.1128/JCM.06697-11.
29. **Facklam R, Beall B, Efstratiou A, Fischetti V, Johnson D, Kaplan E, Kriz P, Lovgren M, Martin D, Schwartz B, Totolian A, Bessen D, Hollingshead S, Rubin F, Scott J, Tyrrell G.** 1999. emm typing and validation of provisional M types for group A streptococci. Emerg. Infect. Dis. **5:**247–253. http://dx.doi.org/10.3201/eid0502.990209.
30. **Bertsch D, Uruty A, Anderegg J, Lacroix C, Perreten V, Meile L.** 2013. Tn6198, a novel transposon containing the trimethoprim resistance gene dfrG embedded into a Tn916 element in Listeria monocytogenes. J. Antimicrob. Chemother. **68:**986–991. http://dx.doi.org/10.1093/jac/dks531.
31. **Coque TM, Singh KV, Weinstock GM, Murray BE.** 1999. Characterization of dihydrofolate reductase genes from trimethoprim-susceptible and trimethoprim-resistant strains of Enterococcus faecalis. Antimicrob. Agents Chemother. **43:**141–147. http://dx.doi.org/10.1093/jac/43.1.141.
32. **Lopez M, Kadlec K, Schwarz S, Torres C.** 2012. First detection of the staphylococcal trimethoprim resistance gene dfrK and the dfrK-carrying transposon Tn559 in enterococci. Microb. Drug Resist. **18:**13–18. http://dx.doi.org/10.1089/mdr.2011.0073.
33. **Rouch DA, Messerotti LJ, Loo LS, Jackson CA, Skurray RA.** 1989. Trimethoprim resistance transposon Tn4003 from Staphylococcus aureus encodes genes for a dihydrofolate reductase and thymidylate synthetase flanked by three copies of IS257. Mol. Microbiol. **3:**161–175. http://dx.doi.org/10.1111/j.1365-2958.1989.tb01805.x.
34. **Dale GE, Langen H, Page MG, Then RL, Stuber D.** 1995. Cloning and

characterization of a novel, plasmid-encoded trimethoprim-resistant dihydrofolate reductase from Staphylococcus haemolyticus MUR313. Antimicrob. Agents Chemother. **39:**1920–1924. http://dx.doi.org/10.1128/AAC.39.9.1920.

35. **Charpentier E, Courvalin P.** 1997. Emergence of the trimethoprim resistance gene dfrD in Listeria monocytogenes BM4293. Antimicrob. Agents Chemother. **41:**1134–1136.
36. **Perreten V, Kadlec K, Schwarz S, Gronlund Andersson U, Finn M, Greko C, Moodley A, Kania SA, Frank LA, Bemis DA, Franco A, Iurescia M, Battisti A, Duim B, Wagenaar JA, van Duijkeren E, Weese JS, Fitzgerald JR, Rossano A, Guardabassi L.** 2010. Clonal spread of methicillin-resistant Staphylococcus pseudintermedius in Europe and North America: an international multicentre study. J. Antimicrob. Chemother. **65:**1145–1154. http://dx.doi.org/10.1093/jac/dkq078.
37. **Kadlec K, Schwarz S.** 2009. Identification of a novel trimethoprim resistance gene, dfrK, in a methicillin-resistant Staphylococcus aureus ST398 strain and its physical linkage to the tetracycline resistance gene tet(L). Antimicrob. Agents Chemother. **53:**776–778. http://dx.doi.org/10.1128/AAC.01128-08.
38. **Kadlec K, Fessler AT, Couto N, Pomba CF, Schwarz S.** 2012. Unusual small plasmids carrying the novel resistance genes dfrK or apmA isolated from methicillin-resistant or -susceptible staphylococci. J. Antimicrob. Chemother. **67:**2342–2345. http://dx.doi.org/10.1093/jac/dks235.
39. **Tanimoto K, Ike Y.** 2002. Analysis of the conjugal transfer system of the pheromone-independent highly transferable Enterococcus plasmid pMG1: identification of a tra gene (traA) up-regulated during conjugation. J. Bacteriol. **184:**5800–5804. http://dx.doi.org/10.1128/JB.184.20.5800-5804.2002.
40. **Sekiguchi J, Tharavichitkul P, Miyoshi-Akiyama T, Chupia V, Fujino T, Araake M, Irie A, Morita K, Kuratsuji T, Kirikae T.** 2005. Cloning and characterization of a novel trimethoprim-resistant dihydrofolate reductase from a nosocomial isolate of Staphylococcus aureus CM.S2 (IMCJ1454). Antimicrob. Agents Chemother. **49:**3948–3951. http://dx.doi.org/10.1128/AAC.49.9.3948-3951.2005.
41. **Cattoir V, Huynh TM, Bourdon N, Auzou M, Leclercq R.** 2009. Trimethoprim resistance genes in vancomycin-resistant Enterococcus faecium clinical isolates from France. Int. J. Antimicrob. Agents **34:**390–392. http://dx.doi.org/10.1016/j.ijantimicag.2009.06.013.