Prevalence of erythromycin and clindamycin resistance among clinical isolates of the *Streptococcus anginosus* group in Germany

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Members of the *Streptococcus anginosus* group (SAG) are frequently involved in pyogenic infections in humans. In the present study, the antimicrobial susceptibility of 141 clinical SAG isolates to six antimicrobial agents was analysed by agar dilution. All isolates were susceptible to penicillin, cefotaxime and vancomycin. However, 12.8% displayed increased MIC values (0.12 mg l⁻¹) for penicillin. Resistance to erythromycin was detected in eight (5.7%) isolates. Characterization of the erythromycin-resistant isolates with the double-disc diffusion test revealed Macrolide-Lincosamide-Streptogramin_B and M-type resistance in six and two isolates, respectively. The erythromycin-resistant isolates were further characterized by PCR for the resistance genes *ermA*, *ermB* and *mefA*. Resistance and intermediate resistance to ciprofloxacin were detected in two and six isolates, respectively. Molecular typing by PFGE revealed a high genetic heterogeneity among the SAG isolates and no evidence for a clonal relationship between the erythromycin-resistant isolates. Our data show that resistance to erythromycin, clindamycin and ciprofloxacin has emerged among SAG isolates in Germany. The implications of these findings for susceptibility testing and antimicrobial therapy of SAG infections are discussed.

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INTRODUCTION

The *Streptococcus anginosus* group (SAG), synonym *Streptococcus milleri* group, is composed of the species *S. anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius* (Facklam, 2002). SAG isolates are part of the normal flora of the human respiratory, gastrointestinal and genitourinary tracts. However, the organisms can also cause serious invasive infections, including infections of the liver and lung, brain abscesses, bacteraemia, endocarditis and intra-abdominal infections (Clarridge *et al.*, 2001; Rashid *et al.*, 2007). In a recent study, members of the SAG were identified as the most frequent cause of invasive pyogenic streptococcal infections in Canada (Laupland *et al.*, 2006).

SAG are generally considered to be susceptible to penicillin, other β -lactam antibiotics and macrolides, but resistant strains have been reported (Aracil *et al.*, 1999; Limia *et al.*,

1999; Tracy *et al.*, 2001). Moreover, a significant increase in the antimicrobial resistance of viridans and β -haemolytic streptococci has been noticed in recent decades (Seppala *et al.*, 2003). The present study was performed to determine the susceptibility *in vitro* of clinical SAG isolates from Germany to penicillin, erythromycin, clindamycin, ciprofloxacin, cefotaxime and vancomycin. Although erythromycin does not represent a first choice antibiotic for the therapy of SAG infections, it was included in this study because resistance to erythromycin may be associated with co-resistance to clindamycin and streptogramins. Macrolide-Lincosamide-Streptogramin_B (MLS_B) resistance has been detected in different bacterial species, including *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Livermore, 2000; Sutcliffe *et al.*, 1996b). The erythromycin-resistant isolates were therefore further characterized with the double-disc diffusion test and PCR to determine the MLS_B resistance phenotype and to identify the erythromycin resistance genes involved. Two main mechanisms are known to account for streptococcal resistance to erythromycin. The MLS_B phenotype is associated with the *erm* genes, which

Abbreviations: MLS_B, Macrolide-Lincosamide-Streptogramin_B; SAG, *Streptococcus anginosus* group; UPGMA, unweighted pair group method with arithmetic averages.

induce the methylation of a ribosomal protein resulting in reduced affinity between MLS_B antibiotics and the ribosome (Seppala *et al.*, 2003). The other resistance type, the so-called M-type resistance, is caused by the *mefA* gene, which induces the active efflux of erythromycin and other 14- and 15-membered macrolides (Sutcliffe *et al.*, 1996b). All isolates were further subjected to PFGE analysis in order to evaluate the genetic heterogeneity among the SAG isolates and the clonal relationship between the erythromycin-resistant isolates.

METHODS

Bacterial isolates. SAG isolates (141) were consecutively collected from March 2002 until March 2003 in three clinical microbiology laboratories located in different regions of Germany: Hygiene Institute, University of Heidelberg, Heidelberg (*n*=83), Vivantes Kliniken, Berlin (*n*=31), and Labor Enders und Partner, Stuttgart (*n*=27). The isolates were grown from clinical specimens from blood (*n*=8), abscess (35), abdomen (24), wounds (21), mediastinum (2), genitourinary tract (11), lower respiratory tract (7), upper respiratory tract (23) and skin (10). Taken together, 45 isolates (32%) were associated with infections of normally sterile body sites, 45 isolates (32%) were obtained from intra-abdominal sites or wounds and 51 isolates (36%) were grown from body sites usually colonized with normal flora. Only one isolate from each patient was included in this study.

Identification and characterization of SAG isolates. Identification was performed by routine laboratory methods including Gram stain, catalase reaction and biochemical analysis using the Api 20 Strep System (bioMérieux) for all isolates. Twelve isolates were additionally subjected to 16S rRNA gene sequencing for genetic identification. All isolates were further characterized by using the Streptococcal Grouping kit (Oxoid) to determine the Lancefield group antigens.

Antimicrobial susceptibility testing. The MIC of several antibiotics was determined by using the agar dilution technique using Mueller–Hinton agar (Oxoid) with 5% sheep blood. An inocula of 10⁴ c.f.u. per spot was used, as described previously (Schoening *et al.*, 2005). Plates were incubated in the presence of 5% CO₂ at 37 °C. The antimicrobial agents included in this study were penicillin G and erythromycin (Grünenthal), cefotaxime (Sigma), clindamycin (Pharmacia), ciprofloxacin (Bayer) and vancomycin (Lilly). *S. pneumoniae* ATCC 49619 and *S. pyogenes* CCUG 25570 and CCUG 25571 were used as controls. The CCUG strains have defined MIC values for erythromycin (Olsson-Liljequist *et al.*, 1997). Clinical and Laboratory Standards Institute breakpoints (Clinical and Laboratory Standards Institute, 2008) were used for all antimicrobial agents except ciprofloxacin, for which the Deutsches Institut für Normung breakpoints (Deutsches Institut für Normung e. V., 1999) were used.

Double-disc diffusion test. The double-disc diffusion test was performed as described previously (Schoening *et al.*, 2005). Briefly, erythromycin (15 µg) and clindamycin (2 µg) (both from Oxoid) were placed 20 mm apart on Mueller–Hinton agar with 5% sheep blood. Plates were incubated overnight at 37 °C in 5% CO₂ in air. Blunting of the clindamycin inhibition zone proximal to the erythromycin disc was interpreted as the inducible MLS_B resistance type, resistance to both antimicrobials was considered as the constitutive MLS_B resistance type and resistance to erythromycin with susceptibility to clindamycin was assigned to the M-type resistance.

16S rRNA gene sequencing and PCR detection of erythromycin resistance genes. Eight erythromycin-resistant and four erythromycin-susceptible isolates were subjected to 16S rRNA gene sequencing for genetic identification, and to PCR assays for detection of the erythromycin resistance genes *ermA*, *ermB* and *mefA*. Bacterial DNA was isolated with the mini tissue kit (Qiagen). 16S rRNA amplification (PCR product of ~510 bp) and sequencing was performed by using the eubacterial primers 5'-AGA GTT TGA TCM TGG CTC AG-3', 5'-GWA TTA CCG CGG CKG CTG-3' and standard protocols. The primer and PCR conditions for amplification of *ermA* (PCR product of ~210 bp), *ermB* (PCR product of ~640 bp) and *mefA* (PCR product of ~350 bp) have been described previously (Morosini *et al.*, 2003; Sutcliffe *et al.*, 1996a).

Macrorestriction analysis of genomic DNA by PFGE. A fresh overnight culture of each isolate was subjected to PFGE analysis following restriction with *Sma*I (Roche) as described previously (Bartie *et al.*, 2000). Electrophoresis was conducted in a CHEF-DRII chamber (Bio-Rad). The data were analysed by Gelcompar II software (Applied Maths). Cluster analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA). An optimization of 1% and a tolerance of 2% were applied.

RESULTS AND DISCUSSION

Characteristics of the SAG isolates

All isolates were assigned by biochemical methods to the SAG. Twelve isolates were additionally subjected to 16S rRNA gene sequencing for genetic identification. The sequence data confirmed the identification of all isolates as members of the SAG (data not shown). Discrepancies at the species level were observed in five cases, which were assigned by biochemical methods as *S. constellatus* (*n*=2), *S. intermedius* (2) and *S. anginosus* (1) and by 16S rRNA gene sequencing as *S. anginosus* (4) and *S. constellatus* (1). Previous studies based on 16S rRNA sequencing have shown the limitations of biochemical testing for identification of SAG isolates to the species level, but have clearly demonstrated the accuracy of biochemical tests for the assignment of streptococci to the SAG (Claridge *et al.*, 1999). Our data are in accordance with the latter findings and confirm that the assignment of SAG isolates to a distinct species within the SAG by biochemical methods is not reliable.

The Lancefield group antigens C, F and G were detected in 49 (34.8%), 45 (31.9%) and 7 (5%) isolates, respectively. Thirty-nine (27.7%) isolates did not express the grouping antigens A, C, F or G.

In vitro antimicrobial susceptibility results

The *in vitro* susceptibility of 141 clinical SAG isolates to penicillin, cefotaxime, erythromycin, clindamycin, ciprofloxacin and vancomycin was studied by agar dilution to determine the MIC values (Table 1). All isolates were susceptible to penicillin, cefotaxime and vancomycin (Table 2). However, 18 (12.8%) isolates displayed a MIC of penicillin of 0.12 mg l⁻¹, which is the susceptible breakpoint for streptococci other than *S. pneumoniae*.

Table 1. *In vitro* activity of six antimicrobials on 141 SAG isolates from Germany

–, Number of isolates=0.

Antimicrobial	No. of isolates inhibited at the indicated concentration (mg l ⁻¹)										
	≤0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	≥16
Penicillin	8	28	87	18	–	–	–	–	–	–	–
Cefotaxime	–	–	6	32	95	8	–	–	–	–	–
Erythromycin	1	4	37	67	24	–	1	–	1	2	4
Clindamycin	3	12	58	64	2	–	–	–	–	1	1
Ciprofloxacin	–	–	–	1	8	70	54	6	1	1	–
Vancomycin	–	–	–	–	4	120	17	–	–	–	–

Our results are in accordance with studies from the USA and Spain, which did not detect resistance against penicillin or third generation cephalosporines among SAG isolates (Aracil *et al.*, 1999; Tracy *et al.*, 2001). These data indicate that penicillin can still be considered as a first-choice drug for the therapy of SAG infections in Germany. However, the increased penicillin MIC in approximately 13 % of isolates underlines the need for determination of MIC values in isolates associated with severe and systemic infections. The relevance of antimicrobial resistance to the outcome of disease has been widely discussed for β -lactams (Pallares *et al.*, 1998). However, little is known about the impact of reduced susceptibility to penicillin in SAG isolates. It is possible that SAG isolates with decreased *in vitro* susceptibility to penicillin may also display a decreased susceptibility *in vivo*, resulting in the need for higher penicillin doses or a switch to other antimicrobial agents, as has been recommended for severe *S. pneumoniae* infections (File, 2006). Further studies concentrating on the clinical outcome of severe SAG infections are required to address this point.

Erythromycin resistance was detected in eight (5.7 %) isolates by agar dilution. The erythromycin MICs were confirmed by E-test in all erythromycin-resistant isolates (data not shown). The erythromycin resistance rate seen in our study is significantly higher than the resistance rate among SAG isolates from the Netherlands (2.6 %) (Jacobs &

Stobberingh, 1996). It is, however, markedly lower than the resistance rate reported in Spain (17.1 %) (Limia *et al.*, 1999). It is interesting to note that the prevalence of erythromycin resistance is markedly higher among *S. pneumoniae* isolates in Germany. In a recent study, the erythromycin resistance rate among invasive *S. pneumoniae* isolates from 2000 was 15.3 % (Reinert *et al.*, 2002). Because of the high frequency of genetic exchange between different streptococcal species, it is likely that the increased macrolide resistance among viridans streptococci has contributed to an increased macrolide resistance among SAG isolates.

Two isolates (1.4 %) were resistant and six (4.3 %) showed intermediate resistance to ciprofloxacin, suggesting that quinolone resistance has emerged among SAG isolates in Germany. Fluoroquinolones are generally not considered as first-choice antibiotics for the treatment of streptococcal infections. However, they are frequently used for the therapy of purulent, multifactorial and intra-abdominal infections. Our data clearly show that even in ciprofloxacin-susceptible isolates, the ciprofloxacin MICs are very close to the resistance breakpoints and suggest that ciprofloxacin is inadequate for the treatment of SAG infections.

Determination of the erythromycin resistance types

The MLS_B resistance phenotype of the erythromycin-resistant isolates (*n*=8) was analysed with the double-disc

Table 2. Range, MIC₅₀, MIC₉₀ and proportion of susceptible and resistant isolates among 141 SAG isolates from GermanyAll values are mg l⁻¹.

Antimicrobial*	Range	MIC ₅₀	MIC ₉₀	% Susceptible	% Resistant
Penicillin (≤0.12; ≥4)	≤0.015–0.12	0.06	0.12	100	0
Cefotaxime (≤0.5; ≥2)	0.06–0.5	0.25	0.25	100	0
Erythromycin (≤0.25; ≥1)	≤0.015–≥16	0.12	0.25	94.3	5.7
Clindamycin (≤0.25; ≥1)	≤0.015–≥16	0.06	0.12	98.6	1.4†
Ciprofloxacin (≤1; ≥4)	0.12–8	0.5	1	94.3	1.4
Vancomycin (≤1; >1)	0.25–1	0.5	1	100	0

*Antimicrobial breakpoints for sensitivity and resistance, respectively, are given in parentheses.

†Isolates of the inducible MLS_B resistance type were not detected as being resistant to clindamycin by means of the MIC values.

Fig. 1. Cluster analysis of PFGE patterns of 119 clinical SAG isolates, as determined with the dice coefficient and the UPGMA method. Erythromycin-resistant isolates have been marked (R).

diffusion test (Arvand *et al.*, 2000; Seppala *et al.*, 1993). Two and four isolates displayed the constitutive and inducible MLS_B resistance phenotypes, respectively. The constitutively MLS_B-resistant isolates displayed MICs of 8–16 mg l⁻¹ for clindamycin, which indicated that they were clindamycin resistant. In contrast, the isolates with inducible MLS_B phenotype displayed clindamycin MICs of 0.06–0.25 mg l⁻¹, which means that they were not recognized as being resistant to clindamycin. Two isolates displayed the M phenotype. All erythromycin-resistant isolates revealed positive PCR results for the erythromycin resistance genes *ermA*, *ermB* or *mefA*. Four isolates with the inducible MLS_B resistance phenotype contained the *ermA* gene. One constitutively MLS_B-resistant isolate contained *ermA* and the second one *ermB* and *mefA*. The two M-type isolates contained the *mefA* gene (data not shown).

Together, six of eight erythromycin-resistant isolates displayed MLS_B resistance and should therefore be considered resistant to clindamycin and Streptogramin_B antibiotics. Hence, the overall frequency of clindamycin resistance was 5% in the present study. This rate is lower than the clindamycin resistance rate (14%) among SAG isolates in the USA (Tracy *et al.*, 2001). Our data indicate that clindamycin-resistant SAG isolates are prevalent in Germany and underline the need for additional testing of the erythromycin-resistant isolates with the double-disc diffusion test to detect inducible clindamycin resistance.

Molecular fingerprinting of the SAG isolates by PFGE analysis

Analysis of the macrorestriction fragments of genomic DNA was performed by PFGE following restriction with *Sma*I. Twenty-two of 141 isolates, including two erythromycin-resistant isolates, did not reveal a sufficient number of bands, i.e. at least six bands, and were therefore considered to be untypable with *Sma*I. The remaining 119 isolates displayed a very high genetic heterogeneity, which is in agreement with the fact that three different species are represented among the SAG isolates. Most isolates displayed restriction patterns that differed by four or more bands from the other isolates. Cluster analysis was performed with the UPGMA method by using the dice coefficient to produce a dendrogram (Fig. 1). A few pairs of isolates displayed indistinguishable or very similar restriction patterns, suggesting a very close genetic relationship. Two erythromycin-resistant isolates (SAG 55 and 150) displayed a similarity of >90% between their restriction patterns, which suggests that they may be clonally related. These isolates were grown from the blood and urine cultures of two different patients that were not epidemiologically related. The remaining four typable erythromycin-resistant isolates displayed different PFGE patterns and were randomly scattered among the SAG isolates. Although we did not obtain sufficient typing results for many isolates (22/141), probably because of the fastidious nature of SAG isolates, the PFGE results of the typable isolates suggest that

the erythromycin-resistant isolates are genetically heterogeneous and not clonally related.

In conclusion, this is the first study to address the antimicrobial susceptibility of SAG isolates in Germany. Although we did not find any penicillin-resistant isolates, the increased penicillin MIC of >0.1 mg l⁻¹ in approximately 13% of the isolates underlines the need for determination of penicillin MIC in SAG isolates associated with invasive or complicated infections. Our data show that erythromycin, clindamycin and ciprofloxacin resistance are prevalent among SAG isolates in Germany and further emphasize the requirement for additional testing of erythromycin-resistant isolates with the double-disc diffusion test in order to detect inducible MLS_B-resistant isolates.

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