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Research

Mutation at the position 2058 of the 23S rRNA as a cause of macrolide resistance in *Streptococcus pyogenes*

Jari Jalava*[1], Martti Vaara[2] and Pentti Huovinen[1]

Address: [1]Department of Human Microbial Ecology and Inflammation, National Public Health Institute, Kiinamyllynkatu 13, 20520 Turku, Finland and [2]Martti Vaara – Division of Clinical Microbiology, Helsinki University Central Hospital, Haartmaninkatu 4, 00029 Helsinki, Finland

Email: Jari Jalava* - jari.jalava@ktl.fi; Martti Vaara - martti.vaara@helsinki.fi; Pentti Huovinen - pentti.huovinen@ktl.fi

* Corresponding author

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Abstract

Background: In streptococci, three macrolide resistance determinants (*erm*(B), *erm*(TR) and *mef*(A)) have been found. In addition, certain mutations at the ribosomal 23S RNA can cause resistance to macrolides. Mutation at the position 2058 of the 23S rRNA of the *Streptococcus pyogenes* as a cause of macrolide resistance has not been described before.

Methods: Antibiotic resistance determinations for the clinical *S. pyogenes* strain ni4277 were done using the agar dilution technique. Macrolide resistance mechanisms were studied by PCR and sequencing. All six rRNA operons were amplified using operon-specific PCR. The PCR products were partially sequenced in order to resolve the sequences of different 23S rRNA genes.

Results: One clinical isolate of *S. pyogenes* carrying an adenine to guanine mutation at the position 2058 of the 23S rRNA in five of the six possible rRNA genes but having no other known macrolide resistance determinants is described. The strain was highly resistant to macrolides and azalides, having erythromycin and azithromycin MICs > 256 microgram/ml. It was resistant to lincosamides (clindamycin MIC 16 microgram/ml) and also MIC values for ketolides were clearly elevated. The MIC for telithromycin was 16 microgram/ml.

Conclusion: In this clinical *S. pyogenes* strain, a mutation at the position 2058 was detected. No other macrolide resistance-causing determinants were detected. This mutation is known to cause macrolide resistance in other bacteria. We can conclude that this mutation was the most probable cause of macrolide, lincosamide and ketolide resistance in this strain.

Background

Streptococcus pyogenes (group A streptococcus) is an important pathogen causing pharyngitis, scarlet fever, impetigo, erysipelas, cellulitis and necrotizing fasciitis. *S. pyogenes* throat infection can lead to suppurative complications like peritonsillar cellulitis and abscesses [1]. Nonsuppurative complications of streptococcal pharyngitis, acute rheumatic fever and glomerulonephritis are rare [2]. *S. pyogenes* can also colonize the throats of asymptomatic persons [1]. The treatment of *S. pyogenes* infections relies on penicillin [3], which has retained its antimicrobial activity and is the drug of choice [4]. Macrolide antibiotics are used to treat patients with allergies to penicillin. In contrast to penicillin, resistance to macrolides among *S. pyogenes* strains has become an increasing problem [5,6].

There are two main acquired macrolide resistance mechanisms in *S. pyogenes*, posttranscriptional target site

modification and macrolide efflux [6]. Target site modification is mediated by erythromycin resistance methylase (*erm*) genes. In *S. pyogenes*, two different Erm methylases have been found. Erm(B) methylase encoded by the *erm*(B) gene belongs to the Erm(B) class of rRNA methylases, and causes resistance to macrolides, lincosamides and streptogramin B antibiotics, i.e. the MLS_B type of resistance. Erm(TR) methylase belongs to the Erm(A) class of rRNA methylases, and also causes the MLS_B type of resistance [7]. However, the type of resistance depends also on the expression type of the Erm methylase. *erm*(B) and *erm*(TR) genes of the inducible type usually cause resistance only to erythromycin and other 14- and 15-membered-ring macrolides but not to lincosamides and streptogramin B antibiotics [5]. After induction of the expression of the gene by sub-inhibitory concentration of erythromycin, these genes cause resistance also to lincosamides and streptogramin B antibiotics [8]. The other main acquired resistance mechanism, macrolide efflux, is mediated by *mef*(A) resistance genes [7]. *mef*(A) genes cause the M type of resistance, i.e. resistance against 14- and 15-membered-ring macrolides [9]. In addition to *mef*(A) mediated macrolide efflux, Giovanetti et al. [10] have demonstrated the presence of a putative novel efflux system, associated with *erm*(TR) gene in *S. pyogenes*. The gene or genes behind this erythromycin efflux system are not yet known.

Mutations at the ribosomal proteins (L4 and L22) and 23S rRNA are known to cause macrolide reistance in streptococci [11,12]. In *S. pyogenes*, mutations at the ribosomal protein L4 and a mutation at the position 2611 of the 23S rRNA as a cause of macrolide resistance have been described before [13,14]. In this work, we describe one clinical *S. pyogenes* strain resistant to macrolides, lincosamides, streptogramin B antibiotics (quinupristin) and telithromycin with none of the known acquired erythromycin resistance methylases or macrolide efflux pumps, but having a mutation at the position 2058 of the 23S rRNA. This mutation has not been described before to cause macrolide resistance in *S. pyogenes*.

Material and methods

Bacterial strains

Macrolide-resistant *Streptococcus pyogenes* strain ni4277 was isolated from a throat specimen in the clinical microbiology laboratory of the Helsinki University Central Hospital. Identification of the strain was based on the colony morphology and hemolysis on Blood Agar Base (Oxoid Ltd., Basingstoke, Hampshire, England) plate supplemented with 7,5% sheep blood as well as resistance to bacitracin (A/S Rosco, Taastrup, Denmark) and positive reaction in group A antigen test (Streptex ZL51, Murex Biotech Ltd., Dartford, England). The identification was further confirmed by sequencing the 23S rRNA genes (see results and discussion). *S. pyogenes* strain ATCC 700294, the whole genome of which has been sequenced, was purchased from the American Type Culture Collection and was used as a macrolide-susceptible control strain. Other bacterial strains with different macrolide resistance determinants were *Escherichia coli* with plasmid pJIR (*erm*(B)), *Staphylococcus simulans* 13044 with plasmid pPV142 (*erm*(C)), *E. coli* with plasmid pEM9592 (*erm*(A)), *S. pyogenes* A200 (*erm*(TR)), *S. pyogens* A569 (*mef*(A)) and *Staphylococcus epidermidis* A33 (*msr*(A)). These strains were used as positive controls in different PCRs [12].

MIC testing and phenotyping

MIC testing was done using the agar-dilution technique [15]. The bacteria were cultured 20 hours in air at 35°C on Mueller-Hinton II (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) agar plates supplemented with 5% sheep blood. The antibiotics used were: clindamycin, josamycin, telithromycin, HMR3004 (Aventis Pharma, Romainville Cedex, France), azithromycin (Pfizer, Groton, CT, USA), quinupristin, spiramycin (Rhône-Poulenc Rorer, Vitrysur-Seine, France), erythromycin, lincomycin (Sigma-Aldrich Chemie, Gmbh, Steinheim, Germany). The double-disk method with erythromycin (diffusible content 78 μg) and clindamycin (diffusible content 25 μg) (Rosco Neo-Sensitabs, A/S Rosco, Taastrup, Denmark) discs was used for classification of macrolide resistance phenotypes. The disks were placed 15–20 mm apart on Mueller-Hinton II agar plates supplemented with 5% sheep blood. Bacteria were cultured 20 hours at 35°C. After incubation, blunting of the clindamycin zone of inhibition proximal to the erythromycin disk was interpreted to indicate inducible MLS_B resistance. NCCLS breakpoints, if available, were used for interpretation of the MIC results [16].

PCR detection of macrolide resistance determinants

The presence of macrolide resistance genes *erm*(B), *erm*(C), *erm*(A), *erm*(TR), *mef*(A) and *mrs*(A) were determined by PCR as described earlier [12]. Amplification of the six ribosomal RNA (rRNA) operons were done using long-range PCRs. Six different PCRs were needed for amplification of six different rRNA operons. The PCRs were identical except for the operon-specific primer. For each rRNA operon, there was one common primer, located at the beginning of the 23S rRNA molecule, and one operon-specific primer (table 1). To obtain high quality DNA for long-range PCR, bacterial cells were cultivated overnight in 10 ml of BHI broth (BD Bioscience, Sparks, MD, USA). Cells were collected by centrifugation and the DNA was isolated as described before [8]. Long-range PCRs were done using DyNazyme Ext DNA polymerase and the buffer supplied with the polymerase (Finnzymes OY, Espoo Finland). The reaction volume was 50 μl, containing 200 ng of the template DNA, 0,5 μM of operon-

Table 1: PCR and sequencing primers

Primer	Target gene	Position	Use	Sequence (5'-3')	Reference
23Scom [a]	23S rRNA	6-26[b]	PCR/Seq	AGTTAATAAGGGCGCACGGTG	This work
23Sg1 [c]	23S rRNA gene1	1577119-157741[d]	PCR/Seq	AAGACGTATTGAAGCTTACTCTA	This work
23Sg2 [c]	23S rRNA gene2	22879-22857 [d]	PCR/Seq	AGCTACTTCCCGAACTGATGCAC	This work
23Sg3 [c]	23S rRNA gene3	29128-29106 [d]	PCR/Seq	GATAACCGTCTTCTTTCCCCTTT	This work
23Sg4 [c]	23S rRNA gene4	85192-85170 [d]	PCR/Seq	CATATTTCTAACACGGGCAGTAG	This work
23Sg5 [c]	23S rRNA gene5	270607-270582 [d]	PCR/Seq	CACTGCCAAGCTATCTAAACGTA	This work
23Sg6 [c]	23S rRNA gene6	1330037-1330059[d]	PCR/Seq	CAATTGAATAGCCTGCACGTTCG	This work
MS2 [e]	23S rRNA	550-573[b]	Sequencing	CGTGTGCCTACAACAAGTTCGAGC	This work
MS3 [e]	23S rRNA	573-550[b]	Sequencing	GCTCGAACTTGTTGTAGGCACACG	This work
MS4 [e]	23S rRNA	1016-997[b]	Sequencing	TTTCGACTACGGATCTTAGC	[11]
MS5 [e]	23S rRNA	867-885[b]	Sequencing	CTGTTTGGGTGAGGGGTCC	[11]
MS6 [e]	23S rRNA	1424-1446[b]	Sequencing	GGACGCAGTAGGCTAACTAAACC	This work
MS7 [e]	23S rRNA	1446-1424[b]	Sequencing	GCTTTAGTTAGCCTACTGCGTCC	This work
MS8 [e]	23S rRNA	2021-2040[b]	Sequencing	ACCTGCATCTTCACAGGTAC	[11]
MS9 [e]	23S rRNA	1902-1921[b]	Sequencing	CGGCGGCCGTAACTATAACG	[11]
MS10 [e]	23S rRNA	2415-2437[b]	Sequencing	GCCATCGCTCAACGGATAAAAGC	This work
MS11 [e]	23S rRNA	2437-2415[b]	Sequencing	GCTTTTATCCGTTGAGCGATGGC	This work
MS12 [e]	23S rRNA	2904-2880[b]	Sequencing	TTGGATAAGTCCTCGAGCTATTAG	[11]
L4fu	L4	64481-64498[c]	PCR/Seq	GCAAACACAGCTCCTAAG	This work
L4rd	L4	65751-65734[c]	PCR/Seq	CTGTGTAACGACCAACGC	This work
L4f156	L4	65074-65091[c]	Sequencing	AACCGTTCAGCAGTATCA	This work
L4r444	L4	65307-65290[c]	Sequencing	TGAAAGGCCTTCTACAGC	This work
L22-1	L22	67079-67098[c]	PCR/Seq	GCAGACGACAAGAAAACACG	[11]
L22-2	L22	67500-67481[c]	PCR/Seq	ATTGGATGTACTTTTTGACC	[11]

[a] A PCR primer common to all 23S rRNA genes [b] Position in the 23S rRNA molecule (*E. coli* accession number J01695, numbering). [c] rRNA operon spesific PCR primers, artificial numbering of the 23S rRNA genes of the *S. pyogenes* [d] Position in the *S. pyogenes* genome (accession number AE004092). [e]23S rRNA gene sequencing primers

specific primer and 0,5 μM of common 23S rRNA primer (table 1), 3 U of the polymerase and 200 μM of each nucleotide. The $MgCl_2$ concentration was 1,5 mM. PCR conditions were as follows: 1 minute at 94°C (one cycle), 20 seconds at 94°C, 30 seconds at 58°C and 3 minutes at 72°C (35 cycles). Amplification was performed using a PTC-200 (MJ Research, Massachusetts, USA). The rRNA operon specific PCR primers were designed using GeneComposer v. 1.1 [17] and the genome sequence of the *S. pyogenes* (accession number AE004092) strain SF370 (same as ATCC 700294) [18].

Amplification of the genes coding for ribosomal proteins L4 and L22 was done using DyNazyme DNA polymerase and buffer supplied with the polymerase (Finnzymes OY, Espoo Finland). The reaction volume was 100 μl, containing 200 ng of the template DNA, 0,2 μM of each primer (table 1), 1 U of the polymerase and 200 μM of the each nucleotide. $MgCl_2$ concentration was 1,5 mM. PCR conditions were as follows: 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C (35 cycles). Amplification was performed using the PTC-200.

Sequencing of the 23S rRNA, L4 and L22 genes

Sequencing was performed using an ABI Prism BigDye Terminator Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction. Sequencing primers are presented in table 1. Sequences were handled using Vector NTi software (InforMax Inc, Bethesda, MD, USA) and the GeneComposer software. Comparisons to bacterial genome sequences were done with the sequence similarity and homology searching tools (Proteomes & Genomes Fasta) using a www-interface to the European Bioinformatics Institute genome database [19].

Accession numbers for the 23S rRNA gene sequences of the strains ni4277 and ATCC 700294, submitted to the EMBL nucleotide sequence database are AJ544681 and AJ544682, respectively. Accession numbers for the genes coding for L4 and L22 proteins of the strains ni4277 are AJ544683 and AJ544684, respectively.

Results

The *S. pyogenes* strain ni4277 was highly resistant to all macrolides and azalides. MICs were: erythromycin >256 μg/ml, spiramycin 64 μg/ml, josamycin 64 μg/ml, azi-

thromycin >256 μg/ml. This strain had elevated MICs for ketolides (telithromycin MIC 16 μg/ml and HMR3004 MIC 4 μg/ml). At the moment, NCCLS does not have breakpoints for telithromycin [16]. However, the high MIC value suggests that this strain is resistant to telithromycin. It was also resistant to lincosamides (clindamycin MIC 16 μg/ml and lincomycin MIC > 64 μg/ml), and it showed elevated MIC for streptogramin B antibiotic, quinupristin MIC 4 μg/ml. When tested with the erythromycin-clindamycin double-disk method, the resistance phenotype in the strain ni4277 was non-inducible. The *S. pyogenes* strain ATCC 700294 was used as a macrolide susceptible control strain. The susceptibility of this strain was confirmed by MIC testing. MICs for various antibiotics were: erythromycin 0,063 μg/ml, spiramycin 0,25 μg/ml, josamycin 0,25 μg/ml, azithromycin 0,125 μg/ml, telithromycin 0,008 μg/ml, HMR3004 0,008 μg/ml, clindamycin 0,031 μg/ml, lincomycin 0,063 μg/ml and quinupristin 0,25 μg/ml. Thus, the MIC values for macrolides, lincosamides, ketolides and quinupristin were much higher in the strain ni4277 than in the strain ATCC 700294. The strain ATCC 700294 has no macrolide resistance determinants in its genome. Neither has it known macrolide-causing mutations in the ribosomal proteins (L4 or L22) or 23S rRNA [18].

In *S. pyogenes*, three macrolide resistance determinants have been described thus far: *erm*(B), *erm*(TR) and *mef*(A) [6]. Using PCR conditions that gave positive results from positive control strains, none of these known macrolide resistance genes was found in the *S. pyogenes* strain ni4277. In addition, PCRs for macrolide resistance genes (*erm*(A), *erm*(C) and *msr*(A)), known to cause resistance in other gram-positive bacteria gave negative results, although PCRs from corresponding control strains were positive.

S. pyogenes has six operons coding for ribosomal RNA molecules [18]. Based on the genome sequence data, primers specific for each rRNA operon were designed (table 1). In order to confirm the specificity of each operon-specific PCR, a short fragment of each PCR product was sequenced using the corresponding operon-specific primer. The sequences were compared to the genome sequence of *S. pyogenes*. These confirmations were done for macrolide-resistant strain ni4277 and for the strain ATCC 700294, the genome sequence of which is available [18]. These sequences confirmed that each operon specific PCR amplified a different ribosomal operon.

The operon-spesific PCRs were used to amplify all the six rRNA operons of the macrolide-resistant strain ni4277. Both DNA strands of the 23S rRNA genes of each six operons were sequenced using 23S rRNA specific sequencing primers (table 1). At least 2854 nucleotides between positions 52 and 2904 of the 23S rRNA gene (*E. coli*, accession number J01695, numbering) from each of the six 23S rRNA coding genes were sequenced. When the six 23S rRNA gene sequences of the different rRNA operons of the strain ni4277 were compared to each other, five of the genes were found to be identical. The remaining 23S rRNA gene differed from the other five genes in one position. All the five 23S rRNA genes had guanine residue at the position 2058 (*E. coli* numbering), whereas adenine residue was found at the corresponding position in the one operon. So, in five of the six 23S rRNA genes of the macrolide-resistant strain ni4277, there was adenine to guanine mutation at the position 2058. This mutation is known to cause macrolide resistance, for example, in *S. pneumoniae* [11]. The sequencing of all the six 23S rRNA genes of the strain ni4277 was done twice. Both times identical sequences were obtained from different operons (five operons with guanine at the position 2058 and one operon with adenine at the corresponding position).

Operon-specific PCRs were also used to amplify 23S rRNA genes of the macrolide-susceptible control strain ATCC 700294. Both DNA strands of all the six 23S rRNA genes of this strain were sequenced. When the sequences (at least 2854 nucleotides between positions 52 and 2904) of these six 23S rRNA genes were compared to each other, they were found to be identical. All the six 23S rRNA genes of this macrolide-susceptible strain had adenine at the position 2058 (*E. coli* numbering). These sequences confirmed that the macrolide-susceptible strain ATCC 700294 has no known macrolide causing mutation at the 23S rRNA, and that the mutations found at the 23S rRNA genes of the strain ni4277 were not caused by a systematic sequencing error.

In addition to the difference at the position 2058 of the 23S rRNA, another difference between the 23S rRNA gene sequences of the macrolide-resistant strain ni4277 and the macrolide-susceptible strain ATCC 700294 was observed. At the position 2163 (*E. coli* numbering) there was a cytosine in all the six 23S genes of the strain ni4277, but thymine at the corresponding position in all the six 23S genes of the strain ATCC 700294. This position is not known to be linked to macrolide resistance [20].

Genes coding for ribosomal proteins L4 and L22 were sequenced, and the sequences were compared against complete genome sequences of *S. pyogenes* using Fasta. Comparisons showed that both ribosomal proteins of the strain ni4277 were identical to the published wild-type L4 and L22 sequences at the amino acid level [18]. No mutations known to cause macrolide resistance in *S. pyogenes* were found [13,14].

Discussion

We studied the presence of efflux pumps in the strain ni4277 by PCR. *mef*(A) or *mrs*(A) genes were not found. This does not exclude, a previously unknown efflux mechanism as the cause of resistance. However, the macrolide resistance phenotype in the strain ni4277 is not typical for efflux pumps, which are rather specific and do not cause resistance against all antibiotics that bind to 23S rRNA [9]. When compared to control strain ATCC 700294, the strain ni4277 had high MIC values for all macrolides, including 14- and 16- membered-ring macrolides, one azalide antibiotic, two different lincosamides, one streptogramin B antibiotic and two different ketolides. These antibiotics have different structures, but they all have common target site, the bacterial ribosome. All these antibiotics bind to or at least are in close contact with the 23S rRNA. So, most likely, target site modification rather than efflux mechanism is the cause of resistance in the strain ni4277.

The *S. pyogenes* strain ni4277 carries two mutations in the 23S rRNA genes (positions 2058 and 2163) when compared to the corresponding genes from a macrolide-susceptible *S. pyogenes* strain ATCC 700294. The other mutated position, 2058, is located at the very conservative part of the domain V of the 23S rRNA molecule. Adenine at the corresponding position can be found in 23S rRNA molecules of almost all bacteria [20,21]. The domain V and the adenine at the position2058 are known to be important for the binding of the macrolides to ribosomes. In bacteria, mutations at this position are always linked to the MLS_B type of resistance or at least erythromycin resistance [20]. The macrolide resistance pattern of the strain ni4277 was of the MLS_B type. It was resistant to macrolides and clindamycin, and it had elevated MIC for streptogramin B (quinupristin) when compared to control strain ATCC 700294. In addition, the strain ni4277 had elevated MIC values for ketolides. In general, the phenotype of the strain ni4277 is very similar to the phenotype of *S. pyogenes* strains with constitutively expressed *erm*(B) methylase: highly resistant to all macrolides, lincosamides, resistant to streptogramin B antibiotics and, the most importantly, resistant to ketolides [5]. This phenotypic similarity supports our finding. If the adenine at the position 2058 is mutated, this mutation produces similar phenotype as if the adenine at the corresponding position were methylated. Erm(B) and erm(TR) methylases as well as other known erythromycin resistance-causing methylases are known to methylate adenine at the position 2058 in streptococci and in other bacteria [5,22]. In the strain ni4277 neither *erm*(B) gene nor any other methylase was found by PCR. Also, it is unlikely that a methylase would be the cause of the resistance in the strain ni4277, because the most of the target sites of the putative methylase were mutated. So the observed mutations at the position 2058 of the 23S rRNA genes are the most likely reason for MLS_B type of resistance and for elevated MICs for ketolides. The other mutated position 2163 is not evolutionary conserved. According to the Comparative RNA Web Site, 31,40%, 13,64%, 52,48% of the bacteria have adenine, cytosine or guanine at this position, respectively, indicating that variation at this position is a normal phenomenon among bacteria [21]. Nor is this position known to interact with macrolides or been associated with macrolide resistance [20]. So it is unlikely that mutation at the position 2163 is the cause of the macrolide resistance.

The strain ni4277 had elevated MICs for ketolides, when compared to the control strain ATCC 700294. Telithromycin MIC 16 μg/ml was so high, that although there are no breakpoints available for telithromycin, this strain is likely to be telithromycin resistant. This finding indicates that a *S. pyogenes* strain can become telithromycin resistant, without acquiring external genetic material. The previously described point mutation, cytosine to thymine, at the position 2611 of the 23S rRNA of *S. pyogenes* [14] does not result in telitromycin resistance. Based on our knowledge, this is the first description of a *S. pyogenes* strain with adenine 2058 to guanine mutation as a cause of macrolide resistance. This strain was isolated from a throat specimen. Thus, our finding also indicates that this kind of mutation in *S. pyogenes* 23S rRNA genes can arise in a clinical situation.

Conclusions

Known acquired erythromycin resistance methylases or macrolide efflux pumps were not found in the *S. pyogenes* strain ni4277. The ribosomal proteins L4 and L22 in this strain were identical to corresponding genes of macrolide-susceptible *S. pyogenes* strain. The macrolide resistance phenotype of this strain was similar to that of other bacteria with adenine at the position 2058 modified or mutated. Based on this data we conclude that the detected mutation, adenine to guanine at the position 2058, in five of the six 23S rRNA-coding genes, was the most probable cause of macrolide resistance in the strain ni4277. Based on our knowledge, this is the first description of this mutation as the cause of macrolide, lincosamide and ketolide resistance in a clinical isolate of *S. pyogenes*.

List of abbreviations

MIC – minimal inhibitory concentration; erm – erythromycin resistance methylase; mef – macrolide efflux; rRNA – ribosomal RNA

Authors' contributions

JJ carried out the MIC and molecular genetic studies. MV carried out the isolation and identification of the strain.

PH participated in the design and coordination of the study. All authors read and approved the final manuscript.

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