Case Report

Macrolide treatment failure in a case of secondary syphilis: a novel A2059G mutation in the 23S rRNA gene of *Treponema pallidum* subsp. *pallidum*

Petra Matějková,¹ Magdalena Flasarová,² Hana Zákoucká,³ Milan Bořek,³ Soňa Křemenová,⁴ Petr Arenberger,⁴ Vladana Woznicová,² George M. Weinstock⁵ and David Šmajs¹

Correspondence

David Šmajs

dsmajs@med.muni.cz

¹Department of Biology, Faculty of Medicine, Masaryk University, Building A6, Kamenice 5, 625 00 Brno, Czech Republic

²Department of Medical Microbiology, Faculty of Medicine, Masaryk University, Pekařská 53, 656 91 Brno, Czech Republic

³National Reference Laboratory for Diagnostics of Syphilis, National Institute of Public Health, Šrobárova 48, 100 42 Prague 10, Czech Republic

⁴Department of Dermatovenereology, Faculty Hospital Královské Vinohrady, Šrobárova 50, 100 34 Prague 10, Czech Republic

⁵Genome Sequencing Center, Washington University School of Medicine, 4444 Forest Park Avenue, St Louis, MO 63108, USA

We report an occurrence of treatment failure after oral spiramycin therapy in a man with secondary syphilis and a reported penicillin and tetracycline allergy. Molecular detection revealed treponemal DNA in the blood of the patient and sequencing of the 23S rDNA identified an A to G transition at the gene position corresponding to position 2059 in the *Escherichia coli* 23S rRNA gene. The occurrence of this novel 23S rDNA mutation was examined among 7 rabbit-propagated syphilitic strains of *Treponema pallidum* and among 22 syphilis patient isolates from the Czech Republic. The prevalence of A2058G and A2059G mutations among clinical specimens was 18.2 and 18.2 %, respectively.

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Introduction

Although parenteral penicillin G is the drug of choice for the treatment of syphilis, an increasing number of patients, with reported allergies to penicillin, require other treatment regimens. The relatively low toxicity and strong bacteriostatic effect of macrolides are reasons for the use of erythromycin and azithromycin for the treatment of the various stages of syphilis.

Unlike penicillin, macrolide regimens represent a risk of treatment failure due to chromosomally encoded resistance in *Treponema pallidum* subspecies *pallidum*. In the year 2000, the macrolide-resistant phenotype of *T. pallidum* subsp. *pallidum* was revealed to be caused by an A2058G (*Escherichia coli* numbering) transition in the 23S rRNA gene (Stamm & Bergen, 2000). Here we describe an A2059G

transition in the treponemal 23S rRNA gene identified in the whole blood sample collected from a patient with secondary syphilis and spiramycin treatment failure.

Case report

A 37-year-old man presented for physical examination at the Department of Dermatovenereology outpatient centre due to a skin and mucosa efflorescence that had persisted for 2 weeks. The patient reported that he was a man having sex with men.

Physical examination revealed a macular rash on the lateral aspects of the trunk, and a psoriasis-like manifestation on the medial aspects of both arms and on the scalp. The oral mucosa was congested, but without visible erosions. The patient's medical records included hepatitis B virus infection, and a reported allergy to penicillin and tetracycline. His human immunodeficiency virus status was examined with negative results.

According to public health law in the Czech Republic, a diagnosis of syphilis needs to be verified by at least one

Abbreviations: CDC, Centers for Disease Control and Prevention; FTA-ABS, fluorescent treponemal antibody absorption; RPR, rapid plasma reagin; SPHA, solid phase haemadsorption; TP-PA, *Treponema pallidum* particle agglutination.

A table of primer sequences is available as supplementary data with the online version of this paper.

treponemal and one non-treponemal test. Positive results in these tests are further confirmed and more precisely specified by additional treponemal tests. Serological diagnostics in this report included the rapid plasma reagin (RPR) test, *T. pallidum* particle agglutination (TP-PA) test, fluorescent treponemal antibody-absorption (FTA-ABS) IgG test, FTA-ABS IgM test and 19S IgM solid phase haemadsorption (SPHA) test.

Molecular detection of treponemal DNA is an additional test that allows identification of macrolide-resistance-causing mutations in clinical isolates. A sample of whole blood was used for this detection. A nested PCR protocol amplifying two different loci [*polA* (Liu *et al.*, 2001) and *tmpC* (Flasarova *et al.*, 2006)] of the *T. pallidum* subsp. *pallidum* chromosome was used as a routine procedure for detecting treponemal genetic material in clinical samples. Additional analysis included a *Mbo*II restriction digest assay of the 23S rDNA amplicon (Lukehart *et al.*, 2004) to look for the presence of a macrolide-resistance-causing mutation (Stamm & Bergen, 2000), and molecular typing of TP0136 and TP0548 (Flasarova *et al.*, 2006). These hypothetical genes represent genetically variable regions among syphilitic isolates, i.e. Nichols and SS14 strains differ in 58 and 42 nucleotides, respectively (Matejkova *et al.*, 2008). Serological findings were as follows: RPR 1:64, TP-PA positive, FTA-ABS IgG positive, FTA-ABS IgM positive and 19S IgM SPHA positive (titre 1:8).

Based on the serological findings, and a history of penicillin and tetracycline allergy, macrolide treatment was administered. The spiramycin therapy was started (i) because of the delay of potential penicillin therapy due to a penicillin skin test (and possible penicillin desensitization) and (ii) because molecular detection tests were expected to soon reveal the presence/absence of a macrolide-resistance-causing mutation.

Spiramycin was administered orally, at a dose 2.25 million units daily (in three doses per day), for a period of 4 weeks. The patient was hospitalized for the first 3 days of antibiotic therapy and he reported complete drug compliance. Molecular detection results were obtained after the start of the spiramycin therapy and were as follows: PCR of the whole blood DNA preparation was positive for treponemal *polA*, *tmpC*, 23S rDNA, TP0136 and TP0548 loci. The *Mbo*II digest assay of the 23S rDNA amplicon did not show the presence of an A2058G transition and the treponemes were considered to be macrolide sensitive. Sequencing of TP0136 and TP0548 loci revealed sequences identical to the corresponding genes of the *T. pallidum* subsp. *pallidum* SS14 strain.

A follow-up examination was performed 2 weeks after the conclusion of spiramycin therapy and consisted of a clinical examination and serology testing. Physical findings revealed persisting exanthema. Serological findings were as follows: RPR 1:32, TP-PA positive, FTA-ABS IgG positive, FTA-ABS IgM positive and 19S IgM SPHA positive (titre 1:32). Macrolide treatment failure was considered and the

titre of IgE antibodies against penicillin was examined with negative results. Intramuscular penicillin therapy was started immediately and continued for 4 weeks. Treatment consisted of 1.2 million units of benzathine penicillin G and 0.3 million units of procaine penicillin G per week. This treatment is in compliance with the guidelines of the Czech Society of Dermatology and Venereology, and this regimen represents a long-term proven treatment of syphilis in the Czech Republic.

Physical examination 1 month after the conclusion of penicillin therapy revealed that the exanthema had disappeared and serological findings revealed a significant decrease in RPR (1:2). Other serological results were as follows: TP-PA positive, FTA-ABS IgG positive, FTA-ABS IgM not examined and 19S IgM SPHA negative.

Methods

DNA isolation. DNA from a whole blood sample was isolated using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions.

Molecular detection of treponemal DNA. A nested PCR protocol was used for the detection of *polA* and *tmpC* (Woznicova *et al.*, 2007), to produce the 23S rRNA gene amplicon for the *Mbo*II/*Bsa*I digest, and amplicons for TP0136 and TP0548 sequencing. A table containing the sequences of all the primers used for the molecular detection of *T. pallidum* is available with the online journal (Supplementary Table S1). The PCR mixture (25 µl) was set up with the following final concentrations: 1 × PCR buffer, 50 µM each dNTP and 1 µM each primer. A total of 10 µl of the examined DNA isolate were added to each reaction. To this mixture, 0.05 µl *Taq* polymerase (5000 U ml⁻¹; New England BioLabs) was added. The protocol was identical for all five products and cycling conditions of the first step were as follows: 94 °C (1 min); 94 °C (30 s), annealing step, see Supplementary Table S1 (available with the online journal) for temperature (30 s), 72 °C (1 min 45 s), for 40 cycles; 72 °C (7 min). For the second step of the PCR, 1 µl PCR mixture containing the first step amplicon was used as a template in a standard 50 µl reaction volume and the following cycling conditions were used: 94 °C (1 min); 94 °C (30 s), annealing step, see Supplementary Table S1 (available with the online journal) for temperature (30 s), 72 °C (1 min 15 s), for 40 cycles; 72 °C (7 min).

In the case of the 23S rDNA amplicon, the first PCR step contained three primers, one forward and two reverse primers, with the objective of including both copies of the 23S rRNA gene present in the *T. pallidum* genome. To test if the nested PCR protocol amplifies both copies of 23S rRNA, a set of two XL PCR products simulating clinical isolates bearing one locus with an A2058G mutation and one wild-type copy of 23S rRNA was used as a template for nested PCR detection. Both digested and non-digested bands were present with similar intensity after the *Mbo*II digest of the PCR product, indicating that both 23S rRNA genes were amplified with similar efficiency.

Restriction digest assay. The assay inspecting both mutations included a parallel *Mbo*II and *Bsa*I restriction digest of the 629 bp amplicon (Fig. 1). Negative results in both reactions indicated the presence of wild-type 23S rDNA. An *Mbo*II positive and a *Bsa*I negative result indicates the presence of the A2058G transition, and a *Mbo*II negative and a *Bsa*I positive result indicated the presence of the A2059G transition in 23S rDNA. *In silico* analysis revealed that a positive *Bsa*I digest reaction enables detection of clinical isolates

Fig. 1. Electrophoretic analysis of *Mbo*II and *Bsa*I restriction digest assay of the 23S rDNA amplicon. 1, 1 kb ladder (NEB); 2, 100 bp ladder (NEB); 3a, uncut (629 bp) Nichols strain (wild-type) DNA; 3b, *Mbo*II digest of Nichols strain (wild-type) DNA; 3c, *Bsa*I digest of Nichols strain (wild-type) DNA; 4a, uncut strain SS14 (A2058G) DNA; 4b, *Mbo*II digest (180 + 449 bp) of strain SS14 (A2058G) DNA; 4c, *Bsa*I digest of strain SS14 (A2058G) DNA; 5a, uncut clinical isolate (A2059G) DNA; 5b, *Mbo*II digest of clinical isolate (A2059G) DNA; 5c, *Bsa*I digest (197 + 432 bp) of clinical isolate (A2059G) DNA. Separate detection of the first 23S rRNA locus (Lukehart *et al.*, 2004) was not successful. The *Mbo*II/*Bsa*I digest of the second copy PCR product resulted in a pattern identical to sample 5 (a, b, c).

bearing A2059G and of clinical isolates bearing both of these mutations, A2058G + A2059G, together. The latter case could be differentiated by the *Bsa*XI digest (positive for A2058G + A2059G) from the clinical isolate bearing A2059G (*Bsa*XI digest negative, based on *in silico* analysis, Table 1).

23S rDNA amplicons were subjected to overnight *Mbo*II and *Bsa*I digestion (New England Biolabs) at 37 and 50 °C, respectively. Resulting fragments were analysed using 2 % agarose gel electrophoresis. Simultaneously, treponemal amplicons were sequenced to confirm the nucleotides at positions corresponding to sites 2058 and 2059 of the *E. coli* rRNA gene.

Molecular typing of clinical isolates. PCR products of TP0136 and TP0548 were purified using a QIAquick PCR purification kit

Table 1. Restriction digest analysis for the detection of mutations in the 23S rRNA gene

Sequence in 23S rDNA	Size of fragment (bp)		
	<i>Mbo</i> II digest	<i>Bsa</i> I digest	<i>Bsa</i> XI digest
Wild-type	629	629	629
A2058G	449 + 180	629	629
A2059G	629	432 + 197	629
A2058G + A2059G*	629 bp†	432 + 197†	429 + 170 + 30†

*Clinical isolate bearing simultaneous mutations at positions 2058 and 2059 has not been described in *T. pallidum*.

†Restriction digest results are based on *in silico* analysis.

(Qiagen) and subjected to dideoxyterminator sequencing using the amplification primers and 4 and 2 additional internal primers, respectively. Sequence analysis was performed using Lasergene software (DNASTAR).

Clinical isolates of *T. pallidum*. A set of 28 clinical isolates with detectable genetic material was collected from 22 patients (17 males and 5 females) in the Czech Republic in the time period 2005–2008. The isolates included 19 lesion swabs, 8 whole blood samples and 1 cerebrospinal fluid sample. The patient age ranged from 0 to 56 years with a median age of 28.5 years.

Results

After successful penicillin therapy, sequencing of the 23S rDNA amplicon (obtained from the sample collected before macrolide therapy) was performed and an A to G transition at the position corresponding to nucleotide 2059 of the *E. coli* 23S rRNA gene was identified. A to G and A to C sequence changes at this position have been described as causing macrolide resistance in a number of bacteria including *Helicobacter pylori*, *Mycobacterium* sp., *Mycoplasma pneumoniae*, *Streptococcus pneumoniae* and propionibacteria (Vester & Douthwaite, 2001), and were studied further in *Mycobacterium smegmatis* (Pfister *et al.*, 2004), but have never been reported in *T. pallidum* strains or isolates.

This finding together with the failure of spiramycin treatment, in the patient, indicated that the A2059G transition could result in a macrolide-resistant phenotype. Experimental verification of the A2059G transition role in macrolide resistance would require a propagation in rabbit and testing of the corresponding strain. However, the need for living treponemes was first recognized during penicillin therapy, and therefore we were not able to isolate living organisms from clinical material and propagate them in a rabbit system.

In order to support our hypothesis that the newly described A2059G mutation is responsible for spiramycin resistance in *T. pallidum* we decided to perform a retrospective examination of available clinical isolates to see if the described mutation is unique or common among syphilis diagnosed patients in the Czech Republic. Since macrolide-resistant clinical isolates are not of clonal origin (Marra *et al.*, 2006), one can expect that the macrolide-resistance-causing mutation would be present in a number of independent clinical isolates. In contrast, an accidental mutation not related to the macrolide-resistance phenotype should be rare among clinical isolates. To test additional treponemal strains and isolates, we designed a *Bsa*I restriction digest assay to detect the A2059G transition, using similar principles to the *Mbo*II digest of the 629 bp amplicon of 23S rDNA for detection of the A2058G transition described by Lukehart *et al.* (2004).

The *Mbo*II/*Bsa*I assay was tested on a set of seven rabbit-propagated syphilitic strains available in our laboratories (Mexico A, DAL-1, Philadelphia-1, Philadelphia-2, MN-3, Grady, Baltimore-73-1). The control samples included (i)

Nichols DNA (wild-type), (ii) SS14 DNA (with an A2058G mutation in the 23S rRNA gene) and (iii) DNA of a clinical isolate bearing the A2059G transition. All seven type strains were found to contain a sequence identical to the Nichols sequence at the examined positions (wild-type). The amplicons were sequenced to confirm the results. In addition to type strains, screening of 28 clinical sample isolates from 22 patients collected in the Czech Republic (including this case report patient), in the time period 2005–2008, was performed. There was complete concordance of multiple samples collected from the same patient, where applicable. A total of 14 patients (63.6%) were infected with macrolide-sensitive strains of *T. pallidum*. Four patients (18.2%) were diagnosed with infection by a strain bearing the A2058G transition and four patients (18.2%) with a strain bearing the A2059G mutation. No direct epidemiological relationship between the patients with the novel mutation in 23S rDNA was found.

Discussion

Although both azithromycin (Hook *et al.*, 1999, 2002; Kiddugavu *et al.*, 2005; Riedner *et al.*, 2005) and spiramycin (Idsoe *et al.*, 1972) were found to be effective in the treatment of early syphilis in humans, an increasing incidence of syphilis isolates resistant to azithromycin has been reported over the past few years (Lukehart *et al.*, 2004; Marra *et al.*, 2006; Mitchell *et al.*, 2006; Morshed & Jones, 2006). The resistance to macrolides is believed to be a product of the frequent use of macrolide regimens for the treatment and prevention of a number of non-syphilitic infections. This has been supported by a study that did not confirm a clonal origin of a group of 20 macrolide-resistant clinical isolates from Washington (Marra *et al.*, 2006).

Testing of macrolide-resistant syphilis treponemes is limited by the fact that *T. pallidum* cannot be cultured under *in vitro* conditions and thus routine testing is restricted to PCR amplification from samples with detectable genetic material. Methods for the molecular detection of the described A2058G mutation in the chromosomal DNA of *T. pallidum* includes restriction analysis of PCR amplicon (Lukehart *et al.*, 2004) or real-time PCR detection of the mutation based on melting curve analysis (Pandori *et al.*, 2007). However, data on the occurrence of macrolide-resistant isolates in many geographical locations is scarce (Katz & Klausner, 2008), and this could be explained by the fact that molecular detection of syphilis is not routinely carried out.

In vitro testing of antibiotic resistance in the *T. pallidum* subsp. *pallidum* Nichols (wild-type) and SS14 (A2058G) strains, in the rabbit system, showed that the SS14 strain was resistant to erythromycin and roxithromycin (14-member lactone ring macrolides); while the Nichols strain was sensitive to these antibiotics. The same study revealed that both strains were highly susceptible to spiramycin and midecamycin (16-member lactone ring macrolides)

(Stamm *et al.*, 1988). These differences probably reflect the differences in binding and mechanisms of action of 14-member and 16-member lactone ring macrolides (Vester & Douthwaite, 2001), and are in complete agreement with structural studies describing the binding of different macrolide molecules to the ribosomal tunnel in *Mycobacterium smegmatis* (Pfister *et al.*, 2004). The presence of the A2059G mutation in this bacterium led to resistance to 14-membered lactone ring macrolides and to a reduced susceptibility to 16-member lactone ring macrolides (Pfister *et al.*, 2004). Based on these facts we can speculate that different types of macrolide-resistant mutations can be expected in strains of *T. pallidum* subsp. *pallidum*.

The newly found A2059G mutation in the 23S rRNA gene of *T. pallidum*, isolated from a patient who experienced spiramycin treatment failure, and the fact that this mutation was found to cause resistance to macrolides in several other bacteria (Vester & Douthwaite, 2001), suggests that the A2059G mutation also causes spiramycin resistance in the syphilis bacterium. The role of A2059G transition in spiramycin resistance is also supported by the fact that the *T. pallidum* subsp. *pallidum* isolates bearing A2059G are not rare in the Czech Republic. Macrolides have been widely used for treatment of respiratory diseases, skin infections and sexually transmitted disease infections (caused by *Chlamydia*) during the last 10 years in the Czech Republic, providing the background for the development of macrolide-resistant bacteria. This fact may explain the relatively high prevalence of clinical isolates bearing transitions A2058G or A2059G.

Our findings contribute to the current discussion regarding the complex situation in worldwide syphilis management, and support Centers for Disease Control and Prevention (CDC) guidelines that highlight the established clinical experience with regard to the use of penicillin G in the treatment of syphilis. Additionally, macrolides should not be used in areas where resistance is widespread and should be used with caution in areas where the prevalence of resistant strains is unknown. In penicillin allergic patients, a penicillin skin test or titre of IgE antibodies against penicillin is required and when positive, desensitization is preferable to the use of non-penicillin regimens (CDC, 2006).

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