

## ORIGINAL ARTICLE

# High frequency of the 23S rRNA A2058G mutation of *Treponema pallidum* in Shanghai is associated with a current strategy for the treatment of syphilis

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The preferred drugs for the treatment of syphilis, benzathine and procaine penicillin, have not been available in Shanghai for many years, and currently, the incidence of syphilis is increasing. Alternative antibiotics for patients with syphilis during the benzathine and procaine penicillin shortage include macrolides. The failure of macrolide treatment in syphilis patients has been reported in Shanghai, but the reason for this treatment failure remains unclear. We used polymerase chain reaction technology to detect a 23S rRNA A2058G mutation in *Treponema pallidum* in 109 specimens from syphilis patients. The use of azithromycin/erythromycin in the syphilis patients and the physicians' prescription habits were also assessed based on two questionnaires regarding the use of macrolides. A total of 104 specimens (95.4%) were positive for the A2058G mutation in both copies of the 23S rRNA gene, indicating macrolide resistance. A questionnaire provided to 122 dermatologists showed that during the penicillin shortage, they prescribed erythromycin and azithromycin for  $8.24 \pm 13.95\%$  and  $3.21 \pm 6.37\%$  of their patients, respectively, and in the case of penicillin allergy, erythromycin and azithromycin were prescribed  $15.24 \pm 22.89\%$  and  $7.23 \pm 16.60\%$  of the time, respectively. A second questionnaire provided to the syphilis patients showed that 150 (33.7%), 106 (23.8%) and 34 (7.6%) individuals had used azithromycin, erythromycin or both, respectively, although the majority did not use the drugs for syphilis treatment. Our findings suggest that macrolide resistance in *Treponema pallidum* is widespread in Shanghai. More than half of the syphilis patients had a history of macrolide use for other treatment purposes, which may have led to the high prevalence of macrolide resistance. Physicians in China are advised to not use azithromycin for early syphilis.

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## INTRODUCTION

Syphilis has increased rapidly in China in the last decade. In 2013, 444 952 cases were reported, with an incidence of 32.86 cases per 100 000 individuals.<sup>1</sup> Shanghai is among the areas with the highest reported incidence, and from 2003 to 2013, 153 716 syphilis cases were presented, of which more than half of the patients (77 931 cases) were with high contagious primary or secondary syphilis (35 804 and 42 127 cases, respectively, provided by the Shanghai Center for Disease Control and Prevention (CDC).

*Treponema pallidum* is the limited-cultivable agent of syphilis. Penicillin has remained the preferred drug for the treatment of syphilis in almost all countries since its discovery more than 60 years ago. Alternative antibiotics that are used during a penicillin shortage or for penicillin-allergic patients with syphilis include doxycycline, tetracycline, ceftriaxone or erythromycin, and more recently, azithromycin.<sup>2-4</sup>

However, erythromycin and azithromycin treatment failures have been documented in patients with primary and secondary syphilis and in infants with congenital syphilis.<sup>5-7</sup> Molecular analysis of the SS14 strain isolated from the clinical specimens of these patients revealed the presence of an adenine (A)-to-guanine (G) transition at the position cognate to A2058 in the *Escherichia coli* 23S rRNA gene compared with

the macrolide-sensitive wild-type Nichols strain of *T. pallidum*.<sup>8-9</sup> We and other researchers previously reported the failures of azithromycin in preventing congenital syphilis and maternal syphilis and in treating a substantial number of patients with primary or secondary syphilis in Shanghai.<sup>5-7</sup>

Given the high incidence of contagious primary and secondary syphilis in Shanghai and the treatment failures that have occurred, surveillance of the drug resistance in syphilis in Shanghai is urgently needed. This study aimed to conduct a survey of the use of macrolides by the patients themselves and the drug preference of physicians for syphilis and non-syphilis treatment and its association with the prevalence of macrolide drug resistance in Shanghai.

## MATERIALS AND METHODS

### Patients

We identified patients with primary or secondary syphilis lesions at the Sexually Transmitted Diseases (STD) Institute at Shanghai Skin Disease Hospital during the period from October 2007–October 2009. Primary syphilis was defined as a clinically compatible case characterized by one or more chancres and inguinal lymphadenopathy and a laboratory confirmation of *T. pallidum* spirochetes in a dark-field

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microscopy examination of a lesion or verification via reactive non-treponemal rapid plasma regain (RPR), or toluidine red unheated serum test (TRUST) and treponemal pallidum hemagglutination (TPHA), or *T. Pallidum* particle agglutination (TPPA). Secondary syphilis was defined as a clinically compatible case characterized by a maculopapular rash and in many cases, lymphadenopathy, and a laboratory test showing a reactive RPR, or TRUST and TPHA or TPPA.

### DNA preparation

After obtaining written informed consent from the subjects, swab samples were collected from the primary or secondary syphilis lesions, placed directly into vials containing 500 µL of lysis buffer (10 mM Tris, pH 8.0; 0.1 M ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate) and frozen at -20 °C. Genomic DNA was extracted from all of the samples using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the protocols recommended by the manufacturer.

### Polymerase chain reaction (PCR)-based Sanger sequencing

To detect the 23S rRNA A2058G gene mutation in the swab samples, a PCR-based restriction digestion was performed as previously described.<sup>8</sup> The PCR primers (Table 1) were designed based on the published Nichols strain of the *T. pallidum* genome.<sup>10</sup> PCR amplification of the 23S rRNA gene was performed for the 628-bp amplicon containing the mutation, followed by *Mbo*II (New England Biolabs) digestion of the purified PCR products. The digestion products were separated on 1.5% agarose gels to identify the mutation. By digesting with *Mbo*II, the DNA from SS14 (Street strain 14 of *T. pallidum*) produces two fragments of 440 bp and 188 bp (Table 1). The wild-type Nichols strain is macrolide-susceptible and does not carry the mutation. To confirm the genotype, the PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and the DNA sequencing of the purified PCR products was performed on an ABI PRISM 377-96 DNA sequencer (Applied Biosystems Inc., Foster, CA, USA).

To ensure that the DNA from other bacteria was not being amplified from the swabs and to determine if the mutation was present in one or both of the 23S rRNA genes (*rrl1* and *rrl2*) from *T. pallidum*, a nested PCR amplification was developed using genes (*tp0226* and *tp0267*) unique to *T. pallidum*.<sup>8</sup> The primers were designed to span *tp0226* and *rrl1* or *tp0267* and *rrl2* (GenBank accession number: NC\_010741.1). The two 1593-bp amplicons were then used as templates for the amplification of the 628-bp products of the 23S rRNA gene. The sequencing and restriction digestion were performed as described above.

### Questionnaire for the use of macrolides

Based on the fact that antibiotic selection may contribute to the increased macrolide resistance in syphilis patients in the United States<sup>11</sup> and the reports of macrolide treatment failures in patients with primary or secondary syphilis and infants with congenital syphilis in China,<sup>6-7</sup> questionnaires on self macrolide use by the patient and physicians' macrolide-prescribing habits for alternative syphilis regi-

mens and how macrolides were used for syphilis patients were designed to evaluate if antibiotic selection is one of the contributing factors for the increasing macrolide resistance in syphilis patients in China.

### Macrolide prescription questionnaire for physicians

A questionnaire regarding alternative syphilis regimens for was sent to 122 dermatologists in 99 hospitals from October 2009 to May 2010. The use of ceftriaxone, tetracycline, doxycycline, minocycline, erythromycin and azithromycin in the case of penicillin allergy or during penicillin shortage was evaluated. The questions included the following: (i) what percentage of your prescriptions were for erythromycin or azithromycin during periods of penicillin shortage? (ii) what percentage of your prescriptions were for erythromycin or azithromycin in the case of penicillin allergy? (iii) for pregnant women, what percentage of your prescriptions were for erythromycin or azithromycin during periods of penicillin shortage? (iv) for pregnant women, what percentage of your prescriptions were for erythromycin or azithromycin in the case of penicillin allergy?

### Macrolide use questionnaire for the patients

During October 2007-October 2009, a total of 605 syphilis patients were asked to complete a questionnaire regarding their history of macrolide use. The questions included the following: (i) do you know about the macrolide antibiotics azithromycin and/or erythromycin? If the answer is yes to question (i), answer the following questions: (ii) have you ever used azithromycin? If yes, what did you use it for? (iii) have you ever used erythromycin? If yes, what did you use it for?

### Statistical analysis

The risks in those with and without the 23S rRNA mutation were assessed via an odds ratio (OR) with a 95% confidence interval (CI). Student's *t*-test was used to analyze the probability of the dermatologists to prescribe erythromycin and azithromycin in cases of penicillin shortage, and a *P*<0.05 was considered to be significant.

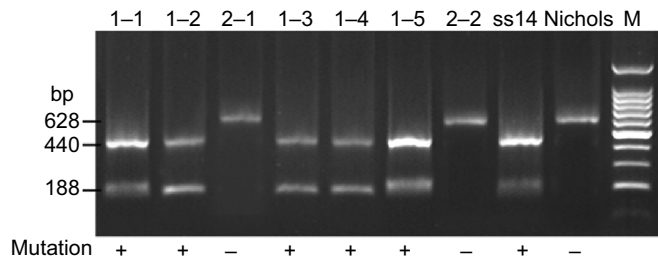
## RESULTS

### The 23S rRNA A2058G mutation in primary and secondary syphilis in Shanghai

During October 2007-October 2009, 182 samples from individuals who were diagnosed with primary or secondary syphilis at two clinics were obtained for molecular analysis, and amplifiable *T. pallidum* DNA was isolated from 109 patients (59.89%). Among the 109 patients, 82 cases were diagnosed as primary syphilis, while 27 were diagnosed with secondary syphilis. The restriction digestion assay showed that 104 samples contained the A2058G mutation in the 23S rRNA genes (95.4%). Figure 1 shows the restriction fragment length polymorphism analysis of the PCR amplicons from the representative samples, suggesting that *T. pallidum* in 104 of the patients was resistant to azithromycin, while the remaining five were sensitive. DNA sequencing of the PCR amplicons confirmed the nucleic acid substitution.

**Table 1** The genes, PCR primers, and expected product sizes in the mutation analysis

Gene target	Primers	Amplicon size (bp)
<i>tp47</i>	F: CGTGTGGTATCA ACTATGG R: TCA ACCGTGTA CTCACTGTC	310
<i>tp0226</i>	F: GTACCGCAAACCGACACAG R: GCGCAACACCTCTTTTAC	1593
<i>tp0267</i>	F: GTACCGCAAACCGACACAG R: GAACCGTCCCTGAAAACCTCA	1593
23S rRNA	F: GTACCGCAAACCGACACAG R: AGTCAAACCGCCACCTAC	628



**Figure 1** The restriction-digestion analysis of the 23S rRNA gene amplicons from two characterized strains of *T. pallidum* (Nichols and Street 14) and from *T. pallidum* samples showing the existence of the A2058G mutation in the majority of the samples.

### The relationship between the 23S rRNA A2058G mutation and macrolide use

A total of 104 patients were infected with macrolide-resistant *T. pallidum*; among them, 40 cases had available macrolide use information, of which only eight (20%), six (15%) and one (2.5%) were treated with azithromycin, erythromycin or both, respectively. For the five patients infected with the Nichols strain, two (40%) of the cases with macrolide use information had never used azithromycin or erythromycin. Table 2 shows the socio-demographic characteristics of these patients.

The above analysis suggests that the relatively high-frequency use of macrolides may have led to the macrolide resistance, although an insufficient number of wild-type *T. pallidum* was obtained in our study because of the widespread macrolide resistance. To further investigate this notion, we surveyed the physicians' prescription habits using a questionnaire. The results showed that a penicillin allergy was the more important factor for determining the drug selection. In the case of benzathine and procaine shortage, and not an allergy, the majority of the dermatologists chose ceftriaxone (data not shown). Additionally, the majority of the dermatologists did not frequently prescribe erythromycin or azithromycin (Table 3). Using descriptive statistics, the data show that the majority of the physicians tended to prescribe erythromycin rather than azithromycin ( $P < 0.001$ ).

Because the above results did not answer our questions, we conducted an additional survey regarding the azithromycin and erythromycin use in syphilis patients of all stages (i.e., primary, secondary, tertiary and latent syphilis) using a macrolide use questionnaire. Overall, 605 syphilis patients were recruited. Out of 445 patients, 150 (33.7%), 106 (23.8%) and 34 (7.6%) had a therapeutic history of azithromycin, erythromycin or use of both, respectively, whereas for the treatment of syphilis, only 23 (3.8%) used azithromycin and none were prescribed erythromycin.

### DISCUSSION

Shortly after the introduction of erythromycin as a form of therapy in the 1950s, resistance to the drug was observed in bacterial pathogens, and several erythromycin-resistant strains were also found to be cross-resistant to all other macrolides.<sup>12–14</sup> As several clinical trials have demonstrated the successful use of a single dose of azithromycin for

early syphilis and the equivalent efficacy of azithromycin and benzathine penicillin G in treating early syphilis in randomized clinical trials,<sup>15–18</sup> azithromycin is believed to be a more attractive treatment alternative for early syphilis than intramuscular penicillin G because it can be administered orally in a single dose and it is more convenient than standard intramuscular penicillin, especially for those who are allergic to penicillin, when there is a shortage of penicillin or when trained staff and injecting equipments are in short supply. According to the United Kingdom national guidelines on the management of syphilis 2008, azithromycin has been recommended to treat incubating syphilis and for epidemiological treatment (a single, oral 1-g dose).<sup>4</sup> However, failures have been observed in syphilis patients treated with macrolide antibiotics.<sup>5–7,9,19,20</sup>

Although macrolide-resistant *T. pallidum* is known in China<sup>10</sup> and azithromycin treatment failures have been reported in Shanghai,<sup>6,7</sup> the data regarding the use of macrolide antibiotics for the treatment of syphilis and macrolide-resistant *T. pallidum* are limited. There are many unanswered questions in China, such as, what is the proportion of macrolide-resistant *T. pallidum*? What contributes to the occurrence of macrolide-resistant *T. pallidum*? What percentage of people is routinely treated with macrolide antibiotics? Why were individuals with syphilis given macrolide antibiotics?

According to the China CDC STD treatment guidelines,<sup>3</sup> the standard and first choice for syphilis is penicillin, and alternative antibiotics, including erythromycin and azithromycin, are allowed for treating syphilis cases only when penicillin is not available or when the patient is allergic to penicillin. However, since the beginning of 2006, there has been a constant shortage of benzathine penicillin and procaine penicillin in Shanghai, and in most hospitals, these two forms of penicillin have been not available for years. Therefore, the alternative antibiotics for syphilis were used during this period.

In this study, 122 dermatologists from 99 hospitals in Shanghai were recruited and surveyed. Ceftriaxone is the first alternative choice for physicians when penicillin is not available or when the patient is allergic to penicillin (data not shown). The data also showed that the physicians' macrolide antibiotic prescription habits for syphilis vary, and they do not frequently use macrolide antibiotics for syphilis. This result was not what we expected, and it did not correspond with the high prevalence of macrolide-resistant *T. pallidum* in Shanghai. However, an investigation of patients with syphilis told us a different story. Among the patients with information regarding their history of macrolide antibiotic use, although only 5.2% were treated with azithromycin for syphilis, 65.1% had used macrolide antibiotics. In China, macrolides have been widely used to treat many pathogenic bacteria, including *Chlamydia trachomatis*, *Ureaplasma urealyticum*, the non-gonococcal urethritis (NGU), which is one of the most common sexually infectious diseases in China. Azithromycin is recommended as the first choice for treating NGU according to the China CDC STD treatment guidelines,<sup>3</sup> and is also used as a prophylaxis against *Chlamydia* infection in individuals with gonorrhoea. In China, although all antibiotics are prescription drugs, it is not difficult

**Table 2** The socio-demographic characteristics among patients with primary or secondary syphilis ( $n=109$ )

	Mutation (104)	Wild type (5)	OR	95% CI
Male/female	72/32	3/2	1.5	(0.12, 13.71)
Age (average)	41.1	38.6		
Primary/secondary	78/26	4/1	0.75	(0.01, 8.06)

**Table 3** The number of dermatologists who prescribed erythromycin and azithromycin during periods of penicillin shortage

	Erythromycin	Azithromycin
Non-pregnant without allergy	8.24±13.95	3.21±6.37
Non-pregnant with allergy	15.24±22.89	7.23±16.60
Pregnant women without allergy	22.70±33.28	3.36±8.29
Pregnant women with allergy	31.61±38.26	5.51±11.89

to obtain oral antibiotics without a prescription from a pharmacy for all types of bacterial infections.

It is worth noting that 24.8% and 17.5% of the patients had a history of azithromycin and/or erythromycin treatment, whereas only 3.8% and 0%, respectively, were for syphilis. Combining the high percentage of *T. pallidum* mutants, we suggest that the macrolide-resistant *T. pallidum* mutation was induced by macrolide pressure that did not result from treating syphilis, but rather, from the abuse of macrolides for other infections.

It is believed that antibiotic selection may have contributed to the increased macrolide resistance in the United States.<sup>11</sup> In the current study, only a relatively small percentage of the patients had been treated with azithromycin (20%), erythromycin (15%), or both (2.5%). Additionally, some of the patients with wild-type *T. pallidum* had never used azithromycin or erythromycin. More importantly, all five samples containing the wild-type strain were collected in 2007 and at the beginning of 2008, which was a year after the beginning of the penicillin shortage in Shanghai. After that time, all of the clinically obtained *T. pallidum* has been macrolide-resistant. Although the results of the macrolide use questionnaire showed that there were no differences between the two groups because of the lack of cases in the wild-type *T. pallidum* group, it could be interpreted that antibiotic selection may contribute to the increased macrolide resistance in Shanghai. Therefore, syphilis patients who have received macrolide treatment must be closely followed-up with clinical and serological evaluations for treatment efficacy, and if possible, the *T. pallidum* mutation should be identified or macrolides should not be used for the treatment of syphilis in Shanghai.

Currently, the occurrence of macrolide resistance in *T. pallidum* from other parts of China remains largely undocumented. It is believed that there is a high potential for the occurrence of macrolide-resistant *T. pallidum* in Nanjing and Guangxi; however,<sup>21</sup> further studies are needed to fully understand the current macrolide use and its association with drug resistance in China.

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