

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Lukehart SA, Godornes C, Molini BJ, et al. Macrolide resistance in *treponema pallidum* in the United States and Ireland. N Engl J Med 2004;351:154-8.

Macrolide resistance in *Treponema pallidum* in the United States and Ireland

Subjects. We identified patients with primary or secondary syphilis at the San Francisco Department of Public Health STD clinic, the Baltimore City Health Department STD Clinic, and the Guide Clinic (Dublin). After obtaining written informed consent, swab samples were collected from primary or moist secondary syphilis lesions, placed directly into vials containing 500 μ L lysis buffer (10mM Tris pH 8; 0.1M EDTA, pH 8; and 0.5 percent sodium dodecyl sulfate), and frozen at -20°C for shipment to the University of Washington for analysis. In Seattle, *T. pallidum* isolates that had been derived from blood and cerebrospinal fluid by rabbit inoculation were provided for molecular analysis. We reviewed medical records to obtain demographic and medical information about syphilis treatment. Approval for collection of samples and review of medical records was obtained from institutional review boards at each of the study sites.

Historical *T. pallidum* strains. The 18 strains of *T. pallidum* subsp. *pallidum* were originally provided by Drs. Paul Hardy and Ellen Nell (Johns Hopkins University) and James N. Miller (University of California, Los Angeles) or were isolated at the University of Washington, then propagated by rabbit passage. These strains represent organisms collected between 1912 and 1987 from a variety of geographical and anatomical sites. Suspensions of each treponemal strain were collected taking necessary precautions to avoid cross contamination from other strains. Bacteria were concentrated

in a microfuge at 13,000 x g for 30 minutes at 4°C and resuspended in 500 µL lysis buffer, as described above.

Gene sequencing and restriction digestion analysis. Initially, two samples from patients (San Francisco) with clinical failure of azithromycin therapy were examined by DNA sequencing for mutations in the 23S rRNA gene sequence; sequence confirmation was subsequently performed on 6 additional samples. DNA was extracted from swab samples or isolates using QIAmp DNA Mini Kit (QIAGEN Inc. Valencia, CA.) under stringent PCR-clean conditions. PCR amplification of the 23S rRNA gene from the extracted DNA was performed using the following primers: Sense 5'- GTA CCG CAA ACC GAC ACA G and Antisense 5'- AGT CAA ACC GCC CAC CTA C, which were designed based on the published *T. pallidum* Nichols genome. A 100 µL reaction containing 0.8 µM concentration of the above primers, 200 µM concentrations of deoxynucleoside triphosphates (Promega, Madison WI), 10 mM Tris-hydrochloric acid (Tris-HCL, pH 9), 50mM potassium chloride (KCL), 0.1 percent Triton X 100, 1.5mM magnesium chloride (MgCl₂), 5 units of *Taq* polymerase (Promega) and 10 µL of DNA, yielded a 628 bp piece of the 23S rRNA genes using the following PCR cycling conditions: initial denaturation at 93°C for 3 minutes, followed by 45 cycles of 93° for 1 minute, 63°C for 2 minutes, 72°C for 1 minute, and a final extension step of 72° for 10 minutes. Amplicons were purified using QIAquick PCR Purification Kit (QIAGEN Inc.).

For DNA sequencing, the amplicons were cloned into TOPO II T/A cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Double-

stranded plasmid DNA from multiple clones was purified with the QIAGEN Plasmid Mini Kit (QIAGEN, Inc.). Full-automated sequencing in both directions was performed on the inserts by the dye terminator method (Perkin-Elmer, Foster City, CA) according to the manufacturer's instructions with the addition of molecular-grade dimethyl sulfoxide to a 5 percent final concentration. The 628 base pair inserts were sequenced with the M13 Forward (5' GTA AAA CGA CGG CCA G) and the M13 Reverse (5'- CAG GAA ACA GCT ATG AC) primers provided for the TOPO II T/A cloning vector. Sequences were assembled using the CAP sequence assembly program at http://www.infobiogen.fr/services/analyseq/cgi-bin/cap_in.pl

For rapid screening of samples for the identified 23S rRNA gene mutation, a restriction digestion assay was developed. Restriction digestion of the purified amplicons was performed using *Mbo*II enzyme according to the manufacturer's instructions (New England Biolabs, Beverly, MA). Digestion products were separated using 3 percent TBE/EtBr Metaphor agarose gels. Samples without the mutation showed a single band at 628 bp, while those with the identified mutation yielded two restriction fragments of 440 and 188 bp. For confirmation, DNA sequencing was performed on a total of 8 samples identified by the restriction analysis assay; in all cases, the identical mutation was identified.

To be certain that DNA from other bacteria was not being amplified from swabs and to determine whether the mutation was present in one or both of the 23S rRNA genes of *T. pallidum*, a nested PCR amplification was developed using different first-round

antisense primers that are unique to *T. pallidum* genes. These two first round primer pairs yield 1593 base pair amplicons. The first primer pair (Sense 5'- GTA CCG CAA ACC GAC ACA G and Antisense 5'-GCG CGA ACA CCT CTT TTT AC) amplifies the relevant portion of one 23S rRNA gene through a part of the flanking gene TP0226; the second set of primers (Sense 5'- GTA CCG CAA ACC GAC ACA G and Antisense 5'- GAA CCG TCC CTG AAA ACT CA) amplifies the relevant portion of the other 23S rRNA gene through part of the flanking gene TP0267. The PCR conditions are identical to those described except that the annealing temperature was 62°C. For the second step of the nesting PCR, one µL of each 1593 base pair amplicon was then used as template for amplification of the 628 base amplicon as described for sequencing and restriction digestion. Digestion with *Mbo*II confirmed that all samples identified with the mutation had the mutation in both 23S rRNA gene copies.

In vivo studies of azithromycin resistance in *T. pallidum*. Four groups of 3 adult male New Zealand White rabbits were infected intradermally (10^6 *T. pallidum* per site) at 8 sites on their shaved backs with either *T. pallidum* Nichols strain (known to be sensitive to azithromycin) or Street 14 strain (known to contain the identified 23S rRNA mutation). Treatment was initiated for each strain when skin lesions had reached approximately 6-7 mm diameter and contained *T. pallidum* identifiable by darkfield microscopy of aspirated material. Treatment groups included untreated controls; BPG 200,000 U/rabbit (equivalent to 4.8 MU w/w in humans), given IM as a single dose; azithromycin 45 mg/day (equivalent to 1 g/day w/w in humans), given orally once daily for two weeks; or erythromycin 90 mg/day (equivalent to 2 g/day w/w in humans) given orally four times

daily for two weeks. Prior to therapy and daily for two weeks, material was aspirated from one lesion on each rabbit and examined by darkfield microscopy for presence of *T. pallidum*; at least 100 fields were examined from each sample and microscopists were blinded to treatment group of the sample. Venereal Disease Research Laboratory (VDRL) titers were determined on each rabbit 6 weeks following initiation of therapy as an independent measure of therapeutic efficacy. Differences were assessed by analysis of variance; P values <0.05 were considered significant.