Supplementary Appendix

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

SUPPLEMENTARY APPENDIX

Lieberman et al., 2023

Near-universal resistance to macrolides of *Treponema pallidum* in North America

Table of Contents **Page**

Methods 2

Ethical approvals and scope *2*

Sample collection, T. pallidum molecular detection, and genomic DNA extraction *2*

Whole genome library preparation and sequencing *4*

Genome Assembly *4*

Phylogenetic Analysis ***5***

Sanger Sequencing of 23S amplicons *5*

Restriction Length Fragment Polymorphism (RFLP)-based detection of A2058G and A2059G mutations *6*

PCR-based detection of A2058G and A2059G mutations *6*

Prior Reporting *7*

Part A: Alternative antibiotic choices for syphilis 8

Part B: Background to azithromycin use in *T. pallidum* 10

Table S1: Patient Characteristics 13

Table S2: Representativeness of syphilis patients included in analysis 16

Table S3: Annotated bibliography of studies worldwide evaluating genotypic AZ resistance in *T. pallidum* 18

Supplementary References 22

Methods

Ethical approvals and scope

Samples collected in Seattle, WA as part of Studies A and B (Supplementary Data) were conducted at the Seattle King County Sexual Health Clinic and approved by the University of Washington Institutional Review Board (IRB) (STUDY00010265 and STUDY00009493, respectively). Study A included remnant pharyngeal, rectal, and vaginal Aptima swabs from patients with a serological diagnosis of syphilis, while Study B involved the collection of swabs of anogenital lesions and oral mucosa from patients with active syphilis. Study C was a multicenter clinical trial (NCT03637660) conducted at Emory University and the University of Alabama at Birmingham (UAB) that involved collection of oral and lesion swabs prior to treatment with penicillin (UAB IRB, protocol# 300001726). Sample collection for Study D was performed at the San Francisco Department of Public Health under protocol University of California, San Francisco IRB protocol # 16-20056. Study E comprised a single sample collected during routine patient care and was therefore not subject to IRB approval. For study F, remnant clinical specimens were obtained from the UW Molecular Microbiology laboratory. The study was approved by the University of Washington IRB (STUDY00138377). Study G was composed of samples received for *T. pallidum* molecular testing at the British Columbia Centre for Disease Control (BCCDC). Study H included oral/lesion swabs or ocular fluid samples or extracted DNA sent to the United States Centers for Disease Control and Prevention (CDC) for molecular surveillance and diagnostics development activities from BCCDC, University of Alberta (IRB: Pro00071804), Emory University (IRB00089239), St. Louis County Sexual Health Clinic, and Dallas County Health and Human Services. Samples used for public health surveillance are IRB exempt.

Whole genome sequencing of deidentified samples was approved by the University of Washington IRB (STUDY00000408 and STUDY00000885).

Sample collection, T. pallidum molecular detection, and genomic DNA extraction

In Study A, *T. pallidum* genomic DNA was detected using transcription-mediated amplification (TMA) of remnant Aptima swab and urine specimens[1]. Genomic DNA was isolated from 200 µL of TMA-positive specimens using the MagNA Pure Total Nucleic Acid Isolation Kit I (Roche) with external lysis using the MagNA Pure 24 instrument. Elution volume was 50 µL.

For Studies B, C, and D, oral or lesion swabs were collected into 1 mL 10 mM Tris, 0.1 M EDTA, with 0.5% SDS. Genomic DNA was extracted from 200 µL of sample buffer using the QIAamp DNA Mini Kit (Qiagen) with a 100 µL elution volume. *T. pallidum* DNA was detected by *TP47* qPCR[2].

For Study E, fourteen 4 µm tissue sections of an 8 mm by 1 mm core needle biopsy were cut from a paraffin-embedded lymph node and combined into a 1.5 ml sterile plastic tube. Deparaffinization and DNA extraction were performed using the QIAamp DNA formalin fixed paraffin embedded (FFPE) Tissue Kit (Qiagen) per the manufacturer's specifications and eluted in 100 µL. Positive qPCR for *TP47* and Warthin-Starry staining confirmed the presence of *T. pallidum.*

For Study F, DNA was extracted as previously described[3] from remnant FFPE or fresh tissue/body fluids samples submitted to the UW Molecular Microbiology laboratory. Samples were interrogated for the presence of *T. pallidum* by either 1) broad-range bacterial PCR with Sanger sequencing[3,4] and amplicon next-generation sequencing for polymicrobial specimens or

2) a proprietary *T. pallidum*-specific laboratory developed nucleic acid amplification test. Taxonomic identification was established following validated procedures including pathologist case review[5].

For Study G, genomic DNA from samples sent to the BCCDC for *T. pallidum* screening was extracted using the Qiagen Blood and Tissue kit and tested for the presence of *T. pallidum* genomic DNA by real-time PCR[6]. Study H samples sent to the CDC were extracted using the Qiagen QIAmp DNA Mini Kit (Qiagen, Germantown, MD), followed by real-time PCR-based detection of *T. pallidum*[7].

Whole genome library preparation and sequencing

Whole genome sequencing (WGS) was attempted at the University of Washington on most samples from Studies A-F with more than 10 *TP47* copies/μL. Libraries were prepared from gDNA using the KAPA HyperPlus kit with UDI adapters (Roche). Up to 100 ng genomic DNA in 8.25μL was used as input for fragmentation for eight minutes, A-tailed and adapters ligated, amplified with 14 PCR cycles, and cleaned with 0.8X AMPureXP beads (Beckman Coulter). 250 ng from each of three libraries were pooled based on similar treponemal load and were enriched using custom biotinylated probes based on the *T. pallidum* reference sequence NC_010741.1 (NCBI) and the IDT xGen Hybridization and Wash kit with overnight hybridization followed by 14 cycles of post-capture PCR. Post-capture libraries were purified using a 0.8X AMPureXP cleanup. Sequencing was performed on 2x151bp Illumina NextSeq2000 or NovaSeq6000 runs[8,9].

Genome Assembly

Genomes were assembled using previously published method[8,10]. Paired-end reads were adapter and quality trimmed using Trimmomatic v0.35[11], kmer filtered to remove human reads using bbduk v38.86[12], and mapped to the *T. pallidum* SS14 reference genome (NC_021508.1), using Bowtie2 software (version 2.4.1)[13] and A2058G/A2059G variants called by freebayes v1.3.2[14]. Mapped reads were *de novo* assembled to scaffolds with Unicycler (version 0.4.4)[15], gaps filled with reference, and iterative remapping of reads performed prior to cleaning with Pilon v1.23.0[16] and calling final consensus. Genomes were considered near-complete if they had less than 5% missing data. To evaluate the presence of the resistance allele, BAM files from samples that had insufficient sequencing depth to generate a near-complete consensus were manually inspected in Geneious Prime v2023.0.4.

Raw data files and consensus genomes have been deposited to NCBI Sequence Read Archive and GenBank under BioProject PRJNA1038754.

Phylogenetic Analysis

Consensus genomes were masked at repetitive loci[10] and aligned with mafft v7.471[17]. Polymorphic sites were extracted with snp-sites v2.5.1[18] and maximum likelihood phylogeny determined using iqtree v2.0.3[19], employing the automated model finder with ascertainment bias correction and 1000 ultrafast bootstraps. The phylogeny was visualized with ggtree[20] and ggplot in R v4.2.1.

Sanger Sequencing of 23S amplicons

For samples from Studies A-F that had insufficient genome copies to attempt WGS, or did not have coverage of the azithromycin resistance loci, we instead amplified a 628 bp fragment[21] of

the genes encoding the two 23S rRNA copies and performed Sanger sequencing. Up to 8 µL DNA was used as input to a 20 µL PCR reaction that also contained 10 µL 2x CloneAmp HiFi Master Mix (Takara) and 1 µL each 10 µM forward (5-GTACCGCAAACCGACACAG-3) and reverse (5- AGTCAAACCGCCCACCTAC-3) primers. All PCR batches included a no-template control (NTC). PCR was performed on a ProFlex thermocycler (Thermo) with a 2 minute denaturation at 98˚C, followed by 35 cycles of 98˚C/10 sec, 70˚C/15 sec, 72˚C/30 sec, then 5 minutes at 72˚C and cold hold. The presence of a band at ~600 bp was verified by 1.4% agarose gel electrophoresis of 4 µl PCR reaction. No amplification of the NTC was also confirmed. Samples with a visible band were AMPure cleaned at 0.6x and sent for Sanger sequencing (Azenta) with the reverse PCR primer. Sequencing traces were manually reviewed for sufficient quality in Geneious v2023.0.4. Sanger sequencing quality scores have been deposited to NCBI Sequence Read Archive under BioProject PRJNA1038754.

Restriction Length Fragment Polymorphism (RFLP)-based detection of A2058G and A2059G mutations

Study G used a RFLP approach to detect genotypic resistance in *T. pallidum*[6]. The 23S rRNA gene was amplified using HotStarTaq Master Mix (Qiagen) with cycling conditions of 95C incubation (15 min), 45 cycles of 95˚C/1 minute, 63˚C/2 minutes, 72˚C/1 minute, and a final 72˚C elongation for 10 minutes. This generated a 629 bp fragment that was then digested overnight with MboII+BsaXI and a separate reaction containing BsaI; characteristic band sizes correspond to the presence of the different alleles[6].

PCR-based detection of A2058G and A2059G mutations

Ten microliters of extracted DNA from Study H was used in a 25 μL triplex PCR reaction[22] that included 200 nM each forward (5- GACTCTGGACACTGTCTCG-3) and reverse (5- Biotin-TTGACTCCGCCTAACCTGACG-3) primers as well as probes to detect wild type (5- FAM - TGAAGGTTCACGGGGTCTTTCCGT-BHQ-3, A2058G (5- CalRed610 - TGAAGGTTCACGGGGTCTTCCCGT-BHQ-3), and A2059G (5- Quasar670 - AAGGTTCACGGGGTCTCTCCGTCT-BHQ -3) alleles. Magnesium Chloride (4nM) (Applied Biosystems, Waltham, MA), 400uM deoxynucleotide triphosphates (Applied Biosystems, Waltham, MA), and AmpliTaq Gold polymerase (ThermoFisher Scientific, Waltham, MA) was used with an initial hold of 95˚C/10 minutes, followed by 50 cycles of 95˚C for 20 seconds/65˚C for 1 minute, and a final 65˚C hold for 10 minutes.

Prior Reporting

Of the 604 samples, 181 (29.9%) had been included in previous publications ([6,8,9,23,2425]) with independent analyses. These samples are clearly identified in Supplementary Data.

Appendix A: Alternative antibiotic choices for syphilis

Long-acting injectable forms of penicillin are the mainstay of syphilis therapy due to their effectiveness for achieving clinical resolution and prevention of late syphilis. However, increasing syphilis case and limited supply chains have led to recurrent shortages of penicillin globally[26]. Currently, there is a shortage of benzathine penicillin G (BPG) first declared April 26, 2023 in the United States (US)[27] while the discontinuation of procaine penicillin – an alternative therapy for treating patients with neurosyphilis in ambulatory settings – was announced by its sole supplier, Pfizer, on June 13, 2023. With rising demand for treatment, health systems and providers with ongoing supply shortages of BPG have transitioned to alternative oral regimens requiring multiday dosing. For early syphilis, such regimens are more burdensome for patients compared to an injection of BPG, heightening concern for lower completion rates, incomplete treatment, and selection of antibiotic resistant strains.

There are some data on the use of alternative therapies. Oral doxycycline is an alternative regimen for persons who are non-pregnant or not capable of pregnancy[28]. Unfortunately, doxycycline requires twice daily dosing for either 14 or 28 days, depending on syphilis stage. Amoxicillin with or without probenecid is an approved alternative regimen in the United Kingdom[29] and a primary regimen in Japan[30], however is not included as an alternative in the CDC STI Treatment Guidelines[31]. Amoxicillin is also dosed orally but has an even higher pill burden than doxycycline, and in the United States has had regular shortages[32].

Limited clinical studies have demonstrated that ceftriaxone is effective for early syphilis although the optimal duration of therapy has not been rigorously defined. It requires daily parenteral doses, but is nonetheless an alternative treatment for early syphilis with a serological response rate of 90% at six months post-treatment in a randomized trial from China[33]. Limited data suggest ceftriaxone may be useful in neurosyphilis: a daily dose of 1-2 g of ceftriaxone IM or IV achieves CSF concentrations of 0.4 mg/L[34], which is > 160-fold higher than the reported minimum inhibitory concentration (MIC) value of 0.0025 mg/L[35]. In the case that parenteral administration of a cephalosporin is not possible, limited data demonstrate that an oral cephalosporin, cefixime, given twice daily for 10 days for early syphilis showed a serological response rate of 87% in a randomized trial[36].

Several additional oral drug regimens with favorable pre-clinical data are currently in clinical trials. Linezolid demonstrated *in vitro* anti-treponemal activity at concentrations ≥0.25 mg/L[37], with a favorable pharmacokinetic profile for CNS penetration and a history of successful treatment of other bacterial CNS infections[38]. A recent clinical trial concluded that the efficacy of linezolid at a daily dose of 600 mg for 5 days did not meet the non-inferiority criteria compared with BPG and, as a result, this treatment regimen should not be used to treat patients with early syphilis[39]. Additional clinical trials, however, are ongoing or planned in Spain, Peru, and the US to evaluate the efficacy of linezolid for early syphilis using a regimen of 600 mg twice daily for ten days[40], which may be necessary to optimize pharmacokinetics and clinical efficacy. Zoliflodacin, an effective treatment for *N. gonorrhoeae*, may also be effective for syphilis given an MIC of 2 mg/L *in vitro*[35]; however there are no *in vivo* data in humans or animal models of syphilis.

Recently, Hayes *et al*. published a high throughput *in vitro* screening of nearly 100 β-lactams that identified mezlocillin, cefmetazole, nafcillin, and azlocillin as the most promising compounds that could be further explored[41]. An antibiotic screening performed *in vitro* by Tantalo *et al*. provided evidence that carbapenems are largely ineffective and reiterated that amoxicillin, cephalosporins, doxycycline, and oxazolidinones are among the most promising candidates to be selected for future clinical trials to evaluate efficacy against syphilis[35].

After more than 80 years of antibiotic treatments for syphilis, penicillin remains first line therapy and the only option for pregnant patients and neonates. Although several effective alternatives are available and others are undergoing clinical trials, all generally require multidose therapy given over days to weeks. Few alternative drugs have robust data supporting effectiveness in neurosyphilis.

Appendix B: Background to Azithromycin Use in *T. pallidum*

Although azithromycin (AZ) has demonstrated effectiveness in early syphilis with an oral single dose regimen, resistance to AZ is now widespread. Macrolides such as AZ inhibit bacterial protein synthesis by binding to the 50S ribosome and preventing exit of the nascent polypeptide. In *T. pallidum*, resistance is conferred by either of two single point mutations in the gene encoding the 23S ribosomal RNA subunit, A2058G or A2059G (in canonical *E. coli* numbering). The presence of either mutation renders the antibiotic unable to bind. Because *T. pallidum* is difficult to isolate from clinical samples and cannot be grown in axenic culture, phenotypic macrolide resistance testing cannot be performed using traditional clinical microbiologic methods. However, molecular detection of either mutation is 100% predictive of macrolide resistance[42],[43].

Historically, macrolide antibiotics were used throughout the world to treat syphilis in persons with penicillin allergies or when access to penicillin was limited[44]. Interestingly, the first documented case of macrolide (erythromycin)-resistance was reported in *T. pallidum* strain SS14[45], which was isolated from a penicillin-allergic patient in 1977. In the 2002 CDC STD Treatment guidelines[46], a single 2g oral dose of azithromycin was discussed as an alternate therapy for syphilis based on preliminary data[47]. Due to its suitability for use in under-resourced settings, AZ treatment was used for patients in Madagascar following an analysis that showed no detection of A2058G among circulating strains, and only a single occurrence of A2059G[48],[42].

However, by the mid-2000s, increasing numbers of treatment failures and genotypic resistance were documented in several geographic locations including the US[21,49] where macrolides were listed as an alternative regimen for those persons with penicillin allergy. AZ resistance may have been further induced in communities by widespread use of azithromycin to prevent sexually transmitted and respiratory infections[50,51]. Given that approximately half of *T. pallidum* strains from the United States tested between 2007 and 2009 harbored AZ resistance alleles[52] and due to documented treatment failures, AZ was no longer recommended as an alternate regimen for syphilis per the CDC STI treatment guidelines[31]. Analysis of the 23S mutations in the context of the worldwide *T. pallidum* phylogeny demonstrated that they arose independently in multiple co-circulating lineages[53]. This observation is consistent with macrolide treatment for any indication within in the prior 12 months being a risk factor for a subsequent syphilis infection with AZ resistance[51].

Recent *in vitro* data have demonstrated that AZ concentrations up to 2 mg/L (64x the MIC for a sensitive strain) are still not effective against cultured *T. pallidum* strains harboring the A2058G or A2059G mutation[35]. Therefore, increasing the azithromycin dose is unlikely to overcome genotypic resistance in syphilis strains and could increase the risk for gastrointestinal side effects and cardiac events[54,55]. These *in vitro* results, combined with the high frequency of resistant *T. pallidum* genotypes and lack of point-of-care clinical resistance testing options, indicate that all syphilis cases should be presumed AZ-resistant.

Table S1: Patient Characteristics. Per category totals include only cases with relevant metadata. Aggregate and per-year data are shown as number (percent) cases per category. AZ resistance of aggregated data is number (percent) resistant per category.

	AZ Resistance n (%) resistant	*All Years n (%) of category total*	2017	2018	2019	2020	2021	2022	2023	Kendall's τ (*p*)
Azithromycin Susceptibility										
Resistant	**599 (100)**	*599 (99.2)*	26 (100)	80 (100)	152 (98.1)	67 (100)	119 (99.2)	81 (98.8)	73 (100)	0.169 (0.738)
Sensitive	**5 (0)**	*5 (0.8)*	0 (0)	0 (0)	3 (1.9)	0 (0)	1 (0.8)	1 (1.2)	0 (0)	0.169 (0.738)
Total	***599 (99.2)***	*604[a]*	*26*	*80*	*155*	*67*	*120*	*82*	*73*	
Of resistant samples, 23S allele										
A2058G	**584 (100)**	*584 (97.5)*	26 (100)	79 (98.8)	146 (96.1)	64 (95.5)	116 (97.5)	81 (100)	71 (97.3)	-0.195 (0.646)
A2059G	**15 (100)**	*15 (2.5)*	0 (0)	1 (1.2)	6 (3.9)	3 (4.5)	3 (2.5)	0 (0)	2 (2.7)	0.195 (0.646)
Total	***599 (100)***	*599*	*26*	*80*	*152*	*67*	*119*	*81*	*73*	
Sex										
Female	**121 (99.2)**	*122 (20.8)*	1 (5.6)	23 (32.1)	29 (19.5)	8 (12.1)	20 (16.7)	15 (18.3)	24 (32.9)	0.333 (0.368)
Male	**460 (99.1)**	*464 (79.2)*	17 (94.4)	53 (67.9)	120 (80.5)	58 (87.9)	100 (83.3)	67 (81.7)	49 (67.1)	-0.333 (0.368)
Total	***586 (99.1)***	*586*	*18*	*76*	*149*	*66*	*120*	*82*	*73*	
Of males, orientation										
MSM	**73 (100)**	*73 (83.0)*	4 (100)	0 (NA)	13 (76.5)	8 (80)	28 (77.8)	18 (94.7)	2 (100)	0.276 (0.566)
MSW	**15 (100)**	*15 (17.0)*	0 (0)	0 (NA)	4 (23.5)	2 (20)	8 (22.2)	1 (5.3)	0 (0)	-0.276 (0.566)
Total	***88 (100)***	*88*	*4*	*0*	*17*	*10*	*36*	*19*	*2*	
Age										
0-17	**5 (100)**	*5 (1.1)*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (2.6)	3 (4.2)	0.724 (0.057)
18-24	**69 (100)**	*69 (15.1)*	1 (7.7)	6 (21.4)	7 (8.2)	5 (7.6)	24 (20.9)	12 (15.4)	14 (19.4)	0.143 (0.764)
25-29	**96 (100)**	*96 (21.0)*	3 (23.1)	3 (10.7)	27 (31.8)	16 (24.2)	25 (21.7)	14 (17.9)	8 (11.1)	-0.333 (0.368)
30-34	**77 (97.5)**	*79 (17.3)*	2 (15.4)	5 (17.9)	11 (12.9)	14 (21.1)	16 (13.9)	16 (20.5)	15 (20.8)	0.333 (0.368)
35-39	**62 (96.7)**	*64 (14.0)*	2 (15.4)	6 (21.4)	6 (7.1)	6 (9.1)	20 (17.4)	10 (12.8)	14 (19.4)	0.143 (0.764)
40-44	**49 (100)**	*49 (10.7)*	2 (15.4)	3 (10.7)	13 (15.3)	8 (12.1)	11 (9.6)	6 (7.7)	6 (8.3)	**-0.714 (0.035)**
45-49	**19 (100)**	*19 (4.2)*	2 (15.4)	2 (7.1)	2 (2.4)	3 (4.5)	5 (4.3)	4 (5.1)	1 (1.4)	-0.524 (0.133)
50-55	**29 (100)**	*29 (6.3)*	1 (7.7)	2 (7.1)	8 (9.4)	2 (3.0)	9 (7.8)	2 (2.6)	5 (6.9)	-0.333 (0.368)
55-59	**19 (100)**	*19 (4.2)*	0 (0)	0 (0)	4 (4.7)	5 (4.7)	3 (2.6)	7 (9.0)	0 (0)	0.309 (0.433)
60+	**27 (96.4)**	*28 (6.1)*	0 (0)	1 (3.6)	7 (8.2)	7 (10.6)	2 (1.7)	5 (6.4)	6 (8.3)	0.429 (0.230)
Total	***452 (98.9)***	*457*	*13*	*28*	*85*	*66*	*115*	*78*	*72*	
Clinical syphilis stage										
Primary	**32 (100)**	*32 (27.8)*	0 (0)	0 (NA)	9 (50.0)	3 (30.0)	15 (26.8)	4 (16.0)	1 (20.0)	-0.200 (0.707)
Secondary	**56 (100)**	*56 (48.7)*	1 (100)	0 (NA)	6 (33.3)	7 (70.0)	26 (46.4)	16 (64.0)	0 (0)	-0.467 (0.260)
Early Latent	**15 (100)**	*15 (13.0)*	0 (0)	0 (NA)	1 (5.5)	0 (0)	10 (17.9)	3 (12.0)	1 (20.0)	0.690 (0.085)
Congenital[b]	**5 (83.3)**	*6 (5.2)*	0 (0)	0 (NA)	0 (0)	0 (0)	1 (1.8)	2 (8.0)	3 (60.0)	**0.894 (0.027)**
Late/ Unk	**6 (100)**	*6 (5.2)*	0 (0)	0 (NA)	2 (11.1)	0 (0)	4 (7.14)4	0 (0)	0 (0)	-0.258 (0.652)
Total	***114 (99.1)***	*115*	*1*	*0*	*18*	*10*	*56*	*25*	*5*	
Race/Ethnicity										
Asian	**4 (100)**	*4 (5.1)*	0 (0)	0 (0)	0 (0)	0 (0)	2 (4.9)	2 (9.5)	0 (0)	-0.316 (0.724)
Black	**22 (100)**	*22 (27.8)*	0 (0)	0 (0)	5 (62.5)	3 (42.9)	12 (29.3)	2 (9.5)	0 (0)	-0.316 (0.724)
Hispanic	**15 (100)**	*15 (19.0)*	0 (0)	0 (0)	0 (0)	0 (0)	10 (24.4)	5 (23.8)	0 (0)	0.359 (0.5791)
White	**33 (100)**	*33 (41.8)*	0 (0)	0 (0)	1 (12.5)	2 (28.6)	16 (39.0)	12 (57.1)	2 (100)	-0.837 (0.096)
NHPI	**1 (100)**	*1 (1.6)*	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.4)	0 (0)	0 (0)	NA
Mixed Race	**4 (100)**	*4 (5.1)*	0 (0)	0 (0)	2 (25.0)	2 (28.6)	0 (0)	0 (0)	0 (0)	-0.800 (0.086)
AI/AN	**0 (0)**	*0 (0)*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
Total	***79 (100)***	*79*	*0*	*0*	*8*	*7*	*41*	*21*	*2*	

State/Province										
Alberta	**100 (100)**	*100 (16.6)*	0 (0)	50 (62.5)	50 (32.3)	0 (0)	0 (0)	0 (0)	0 (0)	
Alabama	**4 (100)**	*4 (0.7)*	0 (0)	0 (0)	0 (0)	0 (0)	3 (2.5)	0 (0)	0 (0)	
BC	**345 (98.9)**	*349 (57.8)*	13 (50.0)	28 (35.0)	75 (48.4)	54 (80.6)	58 (2.9)	51 (62.2)	70 (95.9)	
California	**23 (95.8)**	*24 (4.0)*	12 (46.2)	2 (2.5)	5 (3.2)	0 (0)	3 (2.5)	2 (2.4)	0 (0)	
Colorado	**1 (100)**	*1 (0.2)*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.2)	0 (0)	
CT	**1 (100)**	*1 (0.2)*	0 (0)	0 (0)	0 (0)	1 (1.5)	0 (0)	0 (0)	0 (0)	
DC	**1 (100)**	*1 (0.2)*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.2)	0 (0)	NA[c]
Georgia	**25 (100)**	*25 (4.1)*	0 (0)	0 (0)	9 (5.8)	7 (10.4)	9 (7.5)	0 (0)	0 (0)	
MA	**1 (100)**	*1 (0.2)*	1 (3.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
Minnesota	**2 (100)**	*2 (0.3)*	0 (0)	0 (0)	0 (0)	1 (1.5)	0 (0)	1 (1.2)	0 (0)	
Missouri	**4 (100)**	*4 (0.7)*	0 (0)	0 (0)	1 (0.6)	2 (3.0)	1 (1.3)	0 (0)	0 (0)	
Nebraska	**1 (100)**	*1 (0.2)*	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.3)	0 (0)	0 (0)	
New York	**1 (100)**	*1 (0.2)*	0 (0)	0 (0)	1 (0.6)	0 (0)	0 (0)	0 (0)	0 (0)	
Texas	**15 (100)**	*15 (2.5)*	0 (0)	0 (0)	14 (9.0)	0 (0)	0 (0)	0 (0)	1 (1.4)	
Washington	**74 (100)**	*74 (12.3)*	0 (0)	0 (0)	0 (0)	2 (3.0)	45 (37.5)	25 (30.5)	2 (2.7)	
Wisconsin	**1 (100)**	*1 (0.2)*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.2)	0 (0)	
Total	***599 (99.2)***	*604*	*26*	*80*	*155*	*67*	*120*	*82*	*73*	

NHPI: Native Hawaiian/Pacific Islander. AI/AN: American Indian/Alaska Native. BC: British Columbia. CT: Connecticut DC: District of Columbia. MA: Massachusetts

[a]one additional AZ resistant sample was excluded from the longitudinal analysis due to uncertainty on year of collection (2020 or 2021).

[b]Congenital cases included all cases in infants less than 2 months old (n=5), and a case of syphilis in a 30-34 year old female, with detection of *T. pallidum* DNA in both fetal and maternal tissues

[c]State of collection is study-dependent. Studies were conducted during different time periods.

Table S2: Representativeness of syphilis patients included in analysis. The most recent comprehensive national demographic data from the United States Centers for Disease Control and Prevention, covering the period between 2013-2022[56], and from the Canadian Public Health Agency, covering the period 2011-2020[57], was used to determine representativeness.

Category	Example
Disease, problem, or condition under investigation	Azithromycin resistance in syphilis (all stages)
Special Considerations related to:	
Age	Syphilis is most prevalent among 25-34 year olds for US males and females [56] vs males aged 25-39 and females aged 20-29 in Canada[57]. Cases of congenital syphilis are increased by a factor of ten over the prior decade[56,57].
Sex/gender	Rates of primary and secondary syphilis are highest among individuals designated male at birth in both the US and Canada (~30 per 100,000)[56,57]. Syphilis rates among females have increased rapidly in the last decade to approximately 9 per 100,000 population (United States) and 15 per 100,000 (Canada)[56,57]. The rate of syphilis among MSM (up to 1159 per 100,000) is higher than MSW (~27 per 100,000). MSM still account for the highest number of syphilis cases, but the number of cases in MSW and men with unidentified sex of sex partners has increased more rapidly since 2018[56]. Evidence from a geographically limited study suggests the lifetime prevalence of syphilis among transgender females is twice that of gay males[58].
Race/ethnic group	In the US, the highest rates of syphilis were among American Indian/Alaska Native (AI/AN) persons (67 per 100,000 population in 2022) and Black persons (44.4 per 100,000). Asian and White persons had the lowest rates of syphilis (~5 and 10 per 100,000, respectively)[56].
Geography	Syphilis has increased across all jurisdictions. In Canada, rates have increased most in the prairie provinces, while Nunavut has the highest overall rate of syphilis (127 per 100,000 population, in 2020)[57]. In the US, rates of syphilis are higher in the West and South US Census region than in the Midwest or Northeast[56].
Other considerations	Rates of syphilis among all demographic groups is increasing in the US and Canada[56,57]. Due in part to the low treponemal burden in most stages of syphilis, direct detection of *T. pallidum* DNA has a low clinical sensitivity and is not routinely offered in North American clinical laboratories. Instead, collection of lesion or other swabs for subsequent DNA extraction and molecular testing, including for the presence of azithromycin resistance alleles, is largely confined to a handful of academic medical centers and reference laboratories, including state/provincial and national public health authorities. Reference laboratories frequently do not have access to patient demographic information.

Overall representativeness of this study	Age and sex distribution of patients in this study are similar to that seen in North America: In our study between 2017-2023, females accounted for 20.8% of samples, while in the US in 2022, 24.8% of samples were from females. Among patients in our study, the most common age was 18-24 for females and 25-29 for males[56,57]. We detected a positive temporal trend in the incidence of congenital syphilis, consistent with its increase in incidence across North America. Our study included a larger proportion of MSM (83.0%) among males with partner information compared to syphilis cases across the United States (59.9%)[56]. This may be attributed to sample collection from clinics serving large proportions of MSM. The use of reference laboratory samples with sparse demographic data limits the conclusions we can draw about race/ethnicity: 79/604 (13.1%) of samples in this study included race/ethnicity information and were limited to only certain studies. Among the 79 samples with demographic information, 22 (27.8%) were from Black individuals, 33 (41.8%) from White individuals, and no samples were collected from American Indian/Alaska Native persons. Despite this, the limited data on race/ethnicity roughly reflects US data: In the United States, AI/AN, Black, and White individuals accounted for 2.8, 31.7, and 33.9% of primary and secondary syphilis cases in 2022, respectively[56]. Although our study includes samples from all four US census regions and two Canadian provinces, we do not have any data from most states and provinces.

Table S3: Annotated bibliography of studies worldwide evaluating genotypic AZ resistance in *T. pallidum*.

Years	Location	Macrolide Resistance	Findings	Reference
2004-2017	Czech Republic (Prague and Brno)	**90%**	2016/2017 87% A2058G mutants, 3% A2059G mutants (n=54). Samples collected at two hospitals each in Prague and Brno.	[59]
2005-2020	Australia (multiple locations)	**87%**	87% (398/456) strains were macrolide resistant (allele information not specified). Samples were obtained from the provincial reference laboratory	[60]
2006-2018	The Netherlands (Amsterdam)	**81%**	79% A2058G, 2% A2059G (n=123). Samples collected from an STI clinic in Amsterdam.	[61]
2008-2018	South Africa, Botswana, Zimbabwe	**23%**	23% (22/96) samples AZ resistant via A2058G, with detection only showing up 2013 and onwards. Samples collected from primary health care facilities participating in national STI etiological surveillance programs	[62]
2010-2022	France	**75.6 %**	75.6% (226/299) samples AZ resistant via A2058G. Samples received at national reference center.	[63]
2013-2017	Japan (Osaka)	**86.1%**	86.1% macrolide resistant via A2058G. Resistance in heterosexual groups was 96.6%, while MSM showed 42.9% resistance. Samples collected from STI clinics in Osaka, Japan.	[64]
2014-2018	Japan (Tokyo and Osaka)	**80%**	80% (16/20) strains were macrolide resistant, all via A2058G. Samples collected from four clinics and a hospital.	[65]

2015-2019	Argentina (Buenos Aires)	**45.7%**	45.7% (16/35) macrolide resistant. 93.7% (15/16) A2058G resistant, 1 A2059G mutation found. Samples collected from patients at an STI clinic in Buenos Aires.	[66]
2015-2019	Brazil	**23.5%**	23.5% (16/68) positive for macrolide resistance. 7.4% (5/68) A2058G resistant, 8.8% (6/68) A2059G resistant, 7.4% (5/68) with dual mutation resistance. Samples were from female sex workers	[67]
2016-2017	The Netherlands (Amsterdam)	**88%**	88% (83/93) samples AZ resistant via A2058G. Samples were collected from STI clinics in Amsterdam and general practitioners	[68]
2016-2017	China (Xinjiang)	**100%**	100% (27/27) positive for macrolide resistance, 88.9% (24/27) A2058G resistant, 11.1% (3/27) A2059G resistant. Samples collected from whole blood in latent syphilis patients at the First Hospital of Xinjiang University	[69]
2017	Italy (multiple locations)	**90%**	90% (9/10) strains were macrolide resistant via A2058G. Samples were collected from STI and dermatology clinics	[10]
2017-2018	Japan (Tokyo and Osaka)	**86%**	86% (96/112) A2058G allele, no A2059G. Samples collected from four clinics and a hospital. Samples from MSM were approximately 60% likely to have a resistance allele, while in women and MSM AZ resistance exceeds 95%.	[70]
2017-2018	China (Guangzhou)	**100%**	100% (6/6) strains were macrolide resistant, all via A2058G. Samples were collected from the	[71]

			Dermatology Hospital of Southern Medical University	
2017-2018	United Kingdom (multiple locations)	**94.7%**	94.7% (72/76) strains were macrolide resistant. Of resistant strains, 97.2% (70/72) were resistant via A2058G, 2.8% (2/72) were resistant via A2059G.	[72,73]
2017-2019	Canada (Alberta and British Columbia)	**92.1%**	92.1% (35/38) strains were unambiguously macrolide resistant. 94.3% (33/35) were resistant via A2058G, 5.7% (2/35) were resistant via A2059G.	[73]
2017-2020	Canada (Manitoba)	**99.8%**	99.8% (809/811) macrolide resistant based on A2058G mutation. No A2059G found.	[74]
2018	China (Nanjing)	**100%**	100% (8/8) strains were macrolide resistant via A2058G. Samples were collected from a dermatology clinic.	[10]
2018	Reunion Island	**100%**	100% (7/7) samples AZ resistant via A2058G. Samples collected by the Reunion Island University Hospital	[75]
2018-2019	Cuba (Havana)	**81%**	81% isolates (n=32) were macrolide resistant via A2058G. Samples collected from the Tropical Medicine Institute Pedro Kouri in Havana.	[76]
2018-2019	Hungary (multiple locations)	**50%**	50% (5/10) strains were unambiguously macrolide resistant, all via A2058G.	[73]
2019	Peru (multiple locations)	**77.8%**	77.8% (7/9) strains were macrolide resistant via A2058G. Samples were collected from STI clinics.	[10]
2019	Sweden (multiple locations)	**100%**	100% (7/7) strains were macrolide resistant via A2058G.	[73]
2019-2020	Japan (Tokyo)	**100%**	100% (53/53) strains were macrolide resistant via A2058G. Samples were	[10]

			collected from a dermatology clinic.	
2019-2022	China (Guangzhou)	**100%**	100% (52/52) strains were macrolide resistant, 92.3% (48/52) via A2058G and 7.7% (4/52) via A2059G. Samples were collected from an STI clinic at the Dermatology Hospital of Southern Medical University.	[77]
2019-2022	Malawi (Lilongwe)	**15%**	15% (12/80) strains were macrolide resistant, all via A2058G. Samples were collected from a STI clinic	[78]
2019-2022	Colombia (Cali)	**50%**	50% (13/26) strains were macrolide resistant, all via A2058G. Samples were collected from a public sector healthcare network.	[78]

Pubmed searches were performed between October 20th and November 8th, 2023. Search terms included “syphilis azithromycin”, “syphilis A2058G”, “syphilis macrolide”, “*T. pallidum* azithromycin”, “syphilis whole genome sequencing”. When per sample data were available, dates were filtered to include only samples obtained in 2017 or later. When per sample data were not available, results are limited to those studies that included at least one sample collected since Jan 1, 2017. Datasets with fewer than five samples have been excluded. Studies with any samples included in the current work have been excluded.

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