Selective whole genome amplification as a tool to enrich specimens with low *Treponema pallidum* genomic DNA copies for whole genome sequencing

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Running title: Sequencing of *T. pallidum* from Clinical Specimens

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Abstract.

Downstream next generation sequencing of the syphilis spirochete *Treponema pallidum* subspecies *pallidum* (*T. pallidum*) is hindered by low bacterial loads and the overwhelming presence of background metagenomic DNA in clinical specimens. In this study, we investigated selective whole genome amplification (SWGA) utilizing Multiple Displacement Amplification (MDA) in conjunction with custom oligonucleotides with an increased specificity for the *T. pallidum* genome, and the capture and removal of CpG-methylated host DNA followed by MDA as enrichment methods to improve the yields of *T. pallidum* DNA in rabbit propagated isolates and lesion specimens from patients with primary and secondary syphilis. Sequencing was performed using the Illumina MiSeq v2 500 cycle or NovaSeq 6000 SP platform. These two enrichment methods led to 93-98% genome coverage at 5 reads/site in 5 clinical specimens from the United States and rabbit propagated isolates, containing >14 *T. pallidum* genomic copies/µl input for SWGA and >129 genomic copies/µl for CpG methylation capture with MDA. Variant analysis using sequencing data derived from SWGA-enriched specimens, showed that all 5 clinical strains had the A2058G mutation associated with azithromycin resistance. SWGA is a robust method that allows direct whole genome sequencing (WGS) of specimens containing very low numbers of *T. pallidum*, which have been challenging until now.

Importance

Syphilis is a sexually transmitted, disseminated acute and chronic infection caused by the bacterial pathogen *Treponema pallidum* subspecies *pallidum*. Primary syphilis typically presents as single or multiple mucocutaneous lesions, and if left untreated, can progress through multiple stages with varied clinical manifestations. Molecular studies rely on direct amplification of DNA sequences from clinical specimens; however, this can be impacted by inadequate samples due to disease progression or timing of patients seeking clinical care. While genotyping has provided important data on circulating strains over the past two decades, whole genome sequencing data is needed to better understand strain diversity, perform evolutionary tracing, and monitor antimicrobial resistance markers. The significance of our research is the development of a SWGA DNA enrichment method that expands the range of clinical specimens that can be directly sequenced to include samples with low numbers of *T. pallidum*.

Introduction

Syphilis rates have been steadily increasing in the United States with 38,992 cases (11.9 per 100,000 people) of primary and secondary syphilis and 1,870 cases (48.5 per 100,000 live births) of congenital syphilis reported to the CDC during 2019 (1). This represents a 167.2% increase in primary and secondary syphilis rates since 2010 and a 291.1% increase in congenital syphilis reported since 2015. While syphilis rates have been on the rise in the U.S., the genetic diversity of the bacterial pathogen *Treponema pallidum* subspecies *pallidum* (hereafter referred to as *T. pallidum*), in this setting, is not well understood due to the lack of recently sequenced whole genomes from clinical specimens. Strain diversity has been gleaned from molecular epidemiology studies, which are based on 3 to 4 genetic loci, but may not be representative of the entire *T. pallidum* genome (2-5).

Molecular studies have relied primarily on *T. pallidum* strains propagated in rabbits or DNA amplified directly from clinical specimens, because *T. pallidum* cannot be grown on routine laboratory media. However, advances have been made with *in vitro* tissue culture and the propagation of *T. pallidum* in rabbits from cryopreserved genital lesion specimens, which may make routine culture directly from clinical specimens a possibility in the near future (6-7). Despite these advances, the methods are still time-consuming and impractical for laboratory diagnosis and molecular epidemiological studies of syphilis.

Metagenomic shotgun sequencing approaches have made significant advances in recent years with sequence data being used for pathogen detection, *in silico* or whole genome typing, and antimicrobial resistance marker detection, in addition to phylogenetic analyses (8-10). However, direct whole genome sequencing (WGS) of *T. pallidum* from clinical specimens and rabbit isolates can be problematic due to bacterial genomic DNA being outweighed by either human or rabbit DNA. Several DNA enrichment methods have been described for *T. pallidum* including RNA bait capture techniques, methyl-directed enrichment using the restriction nuclease DpnI, and pooled whole genome amplification, which have generated *T. pallidum* specific WGS data from over 700 metagenomic samples; however, specimens with low numbers of *T. pallidum* remains challenging (11-16). Therefore, additional approaches that would enable sequencing of samples with low bacterial loads are needed.

Azithromycin has been used as an alternative to penicillin for treating early syphilis in the US; however, macrolide-resistant *T. pallidum* strains, associated with two mutations (A2508G, A2509G) in the 23S rRNA genes, have been reported in many states (17-18). While macrolides are no longer recommended for treatment of syphilis in the US, periodic monitoring is useful to determine the prevalence of resistant strains (19).

In this study, we describe a robust DNA enrichment method based on selective whole genome amplification (SWGA) using multiple displacement amplification (MDA) and custom primers that enables WGS of clinical specimens with very low genomic copies of *T. pallidum* and use of the sequence data for macrolide mutation analysis. We also investigated an alternative method that uses CpG methylated capture of host DNA followed by MDA with random oligonucleotide primers.

Results

Real-time qPCR on clinical specimens and spiked samples. The *T. pallidum* PCR results for all clinical specimens are shown in Table 1. Out of the 11 Emory specimens processed using the standard extraction protocol, only one specimen exceeded 100 genomic copies/µl based on *polA* qPCR (Table 1). The remaining 10 specimens had an average copy number <1 copy/µl of DNA extract. These 11 specimens had an average standardized RNP cycle threshold (RNP_{Ct}) value of 30.71 ± 0.13, and the lowest C_{t} value (highest concentration of RNP) was 25.22. Based on this data, an RNP_{Ct} value of 25.22 was targeted as the cut-off for the spiked samples below.

NEBNext microbiome enrichment with MDA. The serially diluted spiked samples enriched with the NEB Microbiome Enrichment Kit with subsequent REPLIg Single Cell MDA (hereafter referred to as NEB+MDA) showed a marked increase in *polA* copy number by qPCR (Fig. 1, Table S1). The non-diluted samples indicated an average *polA* copy number of $6.67 \times 10^{6} \pm 2.74 \times 10^{5}$ per µl of enriched DNA, which was 603.02 times greater than the input copy number. The 10-fold diluted samples also indicated increases in *polA* copy numbers, with an average of $7.85 \times 10^{5} \pm 3.79 \times 10^{4}$, $1.28 \times 10^{5} \pm 1.27 \times 10^{4}$, $8.66 \times 10^{3} \pm 2.54 \times 10^{3}$, and 964 ± 574.23 copies/µl from 1:10 -1:10,000 dilution, respectively (Table S1). This was a 482 – 995.09 times enrichment when compared to the input copy number. Upon comparing the average RNP_{Ct} of each dilution in the series, the enriched samples indicated 29.28 ± 1.07, 31.15 ± 0.46, 30.25 ± 0.56, 31.08 ± 0.59, 31.42 ± 0.45 for the neat – 1:10,000 dilution, respectively (Table S1). The RNP_{Ct} value of each enriched sample in the dilution series were insignificantly different from one another, with an average $\text{RNP}_{\text{Ct}} = 30.64 \pm 0.33$ for all dilutions in the series ($P = 0.22$).

After enriching with NEB+MDA, the average DNA percent for the neat to 1:10,000 dilutions indicated a range of 2.33% ± 0.10- 3.91×10^{-4} % ± 2.50×10^{-4}% of the total DNA belonging to *T. pallidum*, respectively (Fig. 2). Further, this form of enrichment generated up to a 26.12-

fold increase in the percent of *T. pallidum* DNA, and an average of 16.27-fold ± 1.92-fold increase, amongst all enriched replicates when compared to the unenriched input. All samples enriched by NEB+MDA were significantly different in their percent *T. pallidum* DNA when compared to their respective inputs ($P < 0.01$). Apart from enriched samples from the 1:100 and 1:1,000 diluted *polA* inputs, we observed that by increasing the *polA* input copy number 10-fold resulted in a significant increase in the total DNA belonging to *T. pallidum* post-enrichment ($P = 0.06$ and $P < 0.05$, respectively).

Genome sequencing data derived from samples enriched by NEB+MDA showed 0.01 – 10.52% of the quality-controlled reads binned as *T. pallidum*, along with a mean mapping read depth to *T. pallidum* Nichols reference genome *(NC_021490.2)* ranging from 0.05 – 501.75. An average percent coverage of 99.99%, 99.99%, and 97.29% across the Nichols reference genome with at least 5 reads mapped per site (5X) for the neat, 1:10, and 1:100 diluted samples, respectively, was observed among the NEB+MDA enriched samples (Fig.3A; Table S1 and Fig. S1). The coverage estimates indicated low deviations from this average in all replicates, with 2.92×10^{-4} % - 1.38% standard error between all replicates for the neat – 1:100 diluted samples. At the same time, for a higher coverage of at least 10 reads mapped per nucleotide (10X), the 1:100 diluted samples had an average percentage coverage of 84.14% while neat and 1:10 dilution samples were covered at 99.99% and 99.99% across the reference genome, respectively. A sharp decline in coverage was observed in the 1:1,000 diluted samples, with a break down in replication at an average coverage of 27.08% ± 18.62 % for the 1:1,000 dilution and 4.80% ± 0.63% for the 1:10,000 diluted samples at 5X read depth. With the QC criteria for efficiency set at ≥90% at ≥5X read depth, samples sequenced post NEB+MDA enrichment had a limit of detection (LoD) of 129 *polA* copies/µl of extract (Fig. 3A; Table S1 and Fig. S1).

Post NEB+MDA enrichment of isolate CDC-SF003, we observed $2.39 \times 10^{6} \pm 1.35 \times 10^{5}$ *polA* copies/µl of DNA extract. Further, 1.06% of the total DNA belonged to *T. pallidum* post enrichment and 3.29% of the host removed quality-controlled sequencing reads were classified as *T.*

pallidum. Sequencing indicated a 98.60% coverage across the *T. pallidum* SS14 reference genome *(NC_021508.1*) at 5X read depth with a mean mapping depth of 46.43 (Fig. 4; Table 2 and Fig. S2).

SWGA Enrichment of *T. pallidum* Nichols. A total of 12 primer sets were tested by SWGA using Equiphi29 MDA (Table S2-S3). The 1:100 diluted Nichols DNA sample (~129 copies/µl) was used to evaluate each of the 12 primers since it was comparable to specimen EUHM-004, which had 106.7 *polA* copy/µl (Table 1; Table S4). Each of the primer sets indicated a 6.86 – 1.16 x 10^5 times enrichment when compared to the input Nichols copy number (Fig. 5A). Further, we observed a >10,000-fold increase in *polA* copy number in samples enriched with 7 of the 12 primer sets (SWGA Pal 2, 4, 5, 9, 10, 11, and 12). SWGA Pal 9 and Pal 11 gave the highest enrichment at 1.13 x 10^5, and 1.16 x 10^5 times, respectively (Table S4). The difference observed between Pal 9 and Pal 11 in the *T. pallidum polA* copy number and relative percent DNA belonging to *T. pallidum* was insignificant; however, Pal 11 was selected for testing the SWGA limit of detection (P > 0.1; Fig. 5 and Table S4).

To determine the SWGA Pal 11 primer set's LoD and enrichment for *T. pallidum*, SWGA was performed in triplicate on the 10-fold dilution series. The ~1.11x10^4 copies/µl (neat) sample was eliminated from the dilution series, as this was ~100-fold increase in *T. pallidum* copy number when compared to the clinical specimens tested. We observed a marked increase in *polA* copy number in every dilution in the series post enrichment (Fig. 1; Table S1). The *polA* copy number ranged from 1.11 x 10^6 ± 6.68 x 10^5 for the 1:10,000 dilution to 2.04 x 10^7 ± 1.20 x 10^7 in the 1:10 dilution (Table S1). When compared to the input *polA* copy number, this was a 2.01 x 10^4-fold, 1.19 x 10^5-fold, 3.53 x 10^5-fold, and 5.53 x 10^5-fold increase in the enriched samples, from 1:10 -1:10,000 dilution, respectively. Upon comparing the average RNP_{Ct} of each dilution in the series, the SWGA enriched samples indicated a 29.36 ± 0.37 - 28.65 ± 0.16 for the 1:10 -1:10,000 dilution, respectively (Table S1). The average RNP_{Ct} at each 10-fold increase in *polA* concentration were insignificantly different from one another (P > 0.1); however, by increasing the *polA* input 100-fold, we observed a significant decrease in RNP concentration (P < 0.03).

After enriching with SWGA, we observed that dilutions ranging from 1:10 to 1:10,000 held 27.93% ± 1.57% - 3.29% ± 1.93% of the total DNA belonging to *T. pallidum*, respectively (Fig. 2). This reflected up to a 1.63 x 10^5-fold increase in the relative *T. pallidum* and an average of 2.43 x 10^4-fold ± 1.05 x 10^4-fold increase amongst all replicate SWGA enriched samples when compared to the unenriched samples. All samples were significantly increased in their relative *T. pallidum* DNA when compared to their respective inputs ($P < 0.0001$). While there was observed deviations in the percent DNA between replicates, the 1:10,000 diluted replicates still yielded a 28.40-fold ± 17.71-fold increase in DNA belonging to *T. pallidum* post SWGA when compared to the non-enriched neat dilution ($P < 0.0001$).

Genome sequencing data derived from the SWGA enriched Nichols samples showed 0.98%-78.05% of the quality-controlled reads binned as *T. pallidum*, along with a mean mapping read depth to *T. pallidum* Nichols reference genome ranging from 65.82 – 4.89 x 10^3. An average percent coverage of 98.67% ± 0.005%, 98.62% ± 0.003%, and 96.15% ± 0.082% across the Nichols genome at 5X read depth was observed among the SWGA enriched 10-fold dilution series samples for the 1:10, 1:100 and 1:1,000 diluted samples, respectively (Fig. 3B; Table S1 and Fig. S3). Further, coverage indicated low deviations from this average in all replicates, with a 0.0002% - 1.72% standard error between all replicates for the 1:10 – 1:1,000 diluted samples. We did observe a sharp decline in coverage from the 1:1,000 to 1:10,000 dilution with an average coverage of 38.46% ± 2.50% for the 1:10,000 diluted replicates a 5X read depth (Fig. 3B; Table S1 and Fig. S3).

Upon comparing the percent *T. pallidum* DNA derived from both enrichment methods, we observed that SWGA consistently produced higher relative *T. pallidum* DNA in all samples (Fig. 2). We observed that the 10-fold dilutions enriched with SWGA exhibited an average of 94.08-fold - 1.41 x 10^4-fold increase in relative *T. pallidum* DNA in the 1:10-1:10,000 diluted samples when compared to the dilutions enriched by NEB+MDA. All dilutions of each enrichment were significantly different from one another ($P < 0.01$), apart from the 1:10,000 and 1:1,000 diluted samples enriched by SWGA and the neat diluted samples enriched by NEB+MDA ($P > 0.07$).

Comparing the sequencing data derived from the 1:10 and 1:100 diluted Nichols samples enriched using the NEB+MDA and SWGA, all samples exhibited >95% coverage at 5X read depth (Fig. 3; Table S1 and Fig. S1, S3). There was a decline in coverage observed in the 1:1,000 diluted samples enriched by NEB+MDA, with an average coverage of 27.08% ± 24.80% at 5X read depth. This drop was not observed in the 1:1,000 diluted samples enriched by SWGA, which still held >95% coverage at 5X read depth. The 1:10,000 diluted samples enriched NEB+MDA and SWGA exhibited <95% coverage at 5X read depth.

Enrichment of Clinical Strains.

Due to the increased sequencing coverage derived from the SWGA enriched Nichols strain, SWGA was chosen for enriching clinical specimens with low numbers of *T. pallidum* (Fig. 3, Table S1). SWGA on clinical specimen EUHM-004 gave an average *polA* of 6.37 x 10^6 ± 2.24 x 10^5copies/µl with 5.56% of the total DNA belonging to *T. pallidum* (Table 2). Next generation sequencing using the MiSeq v2 (500 cycle) platform revealed 95.13% coverage across the *T. pallidum* genome at 5X read depth (Fig. 4; Table 2 and Fig. S2). After large-scale DNA extraction, we observed 31.5 ± 0.5, 122 ± 1.15, and 103 ± 6.55 *polA* copies/µl for specimens EUHM-012 – EUHM-014, respectively (Table 1). For specimen EUHM-012, we observed an average *polA* of 2.14 x 10^6 ± 2.82 x 10^4 copies/µl with 1.72% of the total DNA belonging to *T. pallidum* post-enrichment by SWGA (Table 2). Sequencing indicated a 93.98% coverage across the *T. pallidum* genome at 5X read depth (Fig. 4; Table 2 and Fig. S2).

When compared to EUHM-012, EUHM-013 had a higher *polA* copy number at 5.16 x 10^6 ± 2.20 x 10^5 copies/µl with 15.48% of the total DNA belonging to *T. pallidum* (Table 2). The sequencing data correlated with the qPCR data, indicating a 98.56% coverage across the *T. pallidum* genome at 5X read depth (Fig. 4; Table 2 and Fig. S2). We also observed EUHM-014 held an increased *polA* copy number post-SWGA, with 2.57 x 10^6 ± 2.21 x 10^5copies/µl and 4.72% of the total DNA belonging to *T. pallidum* (Table 2). Upon sequencing, we observed 98.49% coverage across the *T. pallidum* genome at 5X read depth (Fig. 4; Table 2 and Fig. S2). The *polA* copy number for specimen STLC-001 was 7.42 x 10^6 ± 7.20 x 10^5 copies/µl with 8.34% of the total DNA belonging to *T. pallidum* (Table 2). The sequencing coverage was 95.94% at 5X read depth where 38.91% of the quality-controlled reads binned as *T. pallidum* along with a mean depth read coverage of 1,133.43X (Fig. 4; Table 2 and Fig. S2).

Phylogenetic Analysis and Characterization of Genotypic Macrolide Resistance.

To analyze whether genomes generated from the 7 clinical specimens or isolates clustered to any of the two deep-branching monophyletic *T. pallidum* lineages, Nichols-like and Street-14(SS14)-like, a whole genome phylogenetic tree was constructed using the genomes derived from the clinical specimens/isolates along with 126 high quality published *T. pallidum* genome sequences as of May 2021 (12-15, 20-22; see Table S5, methods in supplemental materials). Phylogenetic analysis revealed the presence of two dominant lineages, of which most strains belonged to the SS14-like lineage. We identified a total of four monophyletic clades within this phylogenetic tree with ≥ 30 bootstrap support (Fig. 6). Three of the clinically derived genomes from Atlanta, EUHM-004 (2019) EUHM-012 (2019), and EUHM-014 (2020), belonged to Nichols-like lineage (clade 1; n=12; Fig. 6). Interestingly, the other nine Nichols-like genomes in clade 1 were recent clinically derived genomes from Cuba (n=2; 2015-2016), Australia (n=1; 2014), France (n=2; 2012-2013) and UK (n=3; 2016), and were distinct from the original Nichols strain isolated in 1912 and sent to different North American labs as *in vivo* derived clones, suggesting that we might not yet fully understand the current diversity of this lineage. The three clinical specimens from Atlanta (EUHM-004, EUHM-012, and EUHM-014) and three clinically derived genomes from UK isolated in 2016 (NL14, NL19 and NL17) carried the 23S rRNA A2058G mutation that confers macrolide resistance, suggesting a recent acquisition of this antibiotic resistance variant in the Nichols-like lineage.

Even though previous phylogenomic analyses indicated that SS14-lineage showed a polyphyletic structure, our phylogenetic analysis with a greater number of genomes showed the presence of 3 monophyletic clades (Clades 2, 3 and 4)(12, 14; Fig. 6). Clades 2 and 4 contained genomes clustered within the previously reported SS14Ω-A sub cluster, which also contained two clades corresponding to the clades 2 and 4 detected in this study, and contained genomes derived from Europe and North America; while clade 3 was similar to sub cluster SS14Ω-B and composed of Chinese and North American derived *T. pallidum* genomes. The rabbit-derived clinical isolate, CDC-SF003 (San Francisco, U.S; 2017) sequenced in this study, clustered within clade 2; while EUHM-013 (Atlanta, U.S; 2020) and STLC-001 (St. Louis, U.S; 2020) genomes clustered within clade 4. Sequence analysis showed that all 3 strains carried the A2058G AMR variant for macrolide resistance. Macrolide resistance strains were widespread among the SS14-lineage with higher proportion among the genomes in clades 2 and 3 compared to clade 4 genomes. The A2058G point mutation identified in 4 patient specimens and isolate CDC-SF003 was verified by real-time PCR testing of genomic DNA and SWGA-enriched samples (data not shown). There was inadequate sample for the fifth specimen to confirm the mutation by real-time PCR testing.

All the Nichols-like genomes derived from the NEB+MDA and SWGA 10-fold dilution series that contained *T. pallidum* reads mapped to ≥90% of the genome with at least 5X read depth formed a tight monophyletic clade (bootstrap support of 88/100) and clustered with the lab-derived Nichols-Houston-J genome (bootstrap support of 100/100), indicating that genomes generated from both methods are adequate to capture genetic variants required to perform a high resolution phylogenetic analysis (Fig. S4).

Discussion

WGS of *T. pallidum* is often challenging due to low bacterial loads or the difficulty of obtaining adequate samples for testing. In this study, we sought to develop a method for performing WGS from rabbit propagated isolates and clinical specimens containing lower *T. pallidum* numbers, leading us to investigate CpG capture and SWGA.

CpG capture has been successfully used for enriching bacterial genomic DNA in metagenomic samples (23-24), but this method has not been used for *T. pallidum*. During our testing, we observed increases in *polA* copy numbers and relative *T. pallidum* percent DNA in the neat to 1:1,000 dilutions enriched by NEB+MDA when compared to the non-enriched inputs. Further, the results of the percent *T. pallidum* observed in the enriched 1:10,000 diluted samples correlated with the decrease in overall coverage across the Nichols genome. Even though we observed an increase in both *polA* copy number and relative percent *T. pallidum* DNA for the enriched diluted 1:1,000 diluted samples, we still only gained ~50% genomic coverage. This could be due to the remnant human DNA that was not initially captured prior to MDA, or the loss of *T. pallidum* DNA during the enrichment. While there was no significant difference in the relative human RNP copy number from dilution to dilution, there is a minimum *T. pallidum* copy number input required to outweigh the remnant human DNA during the metagenomic shotgun sequencing. Taking the above into consideration, we observed that >129 *polA* copies/µl can generate >95% coverage at 5X read depth from the Nichols strain post NEB+MDA. The results observed post NEB+MDA enrichment of clinical isolate CDC-SF003 correlated with the Nichols limit of detection validation, with >98% coverage at 5X read depth across the *T. pallidum* genome. *In silico* variant analysis correlated with real-time PCR detection of the mutations associated with macrolide resistance in clinical isolate CDC-SF003. Further, phylogenetics revealed that this strain belonged to the SS14 lineage, which correlated with its enhanced CDC typing method (ECDCT) strain type, 4d9f, as previously reported (7). While this enrichment method yielded good results with isolates, most clinical specimens collected in this study had lower than 100 *polA* DNA copies/µl of *T. pallidum* leading us to consider an alternative method.

SWGA has been shown to be successful with other bacterial pathogens in metagenomic samples; however, it has not been investigated with *T. pallidum* (25-27). We observed that samples enriched by SWGA using multiple primer sets exhibited a 10,000-fold increase in *polA* copy number, with Pal 9 and 11 producing the highest relative percent *T. pallidum* DNA at 29% and 31%, respectively. While we chose to work with Pal 11 as the optimal set, Pal 9 could also be a good alternative for enriching syphilis specimens. Further testing using Pal 11 showed that the limit of detection was increased when compared to the *T. pallidum* enrichment obtained with NEB+MDA, with significant increases in both *polA* copy number and percent *T. pallidum* across the 10-fold dilution series. Coverage across the *T. pallidum* genome exceeded 95% at 5X read depth for all diluted samples, apart from the 1:10,000 diluted samples. Interestingly, we observed that increasing the input 100-fold resulted in a significant decrease in the presence of RNP post-enrichment. Our data shows that >14 *T. pallidum polA* copies/µl can generate at least 95% coverage at 5X read depth with the Nichols strain, which translated well to the clinical specimens tested. While there was a decrease in coverage in one of the clinical specimens at 94.44% with 5X read depth when compared to the 98.62% coverage at 5X read depth observed in the 1:100 diluted Nichols isolates, this could be primarily due to the improved capabilities of the NovaSeq 6000 when compared to the MiSeq v2 (500 cycle) platform used to sequence this clinical specimen. Another possible reason for the variation in coverage could be due to the lower *T. pallidum* input copy number in the clinical specimens.

The genomes derived directly from the 5 clinical specimens using SWGA were phylogenetically associated with the representative lineages (either Nichols-like or SS14-like) and also provided high levels of within lineage strain resolution, which is ideal for effective tracking of various strains circulating within a geographical area and outbreak investigations. In addition, the NGS methods described here can be used for macrolide resistance marker detection. As observed with NEB+MDA enrichment, *in silico* azithromycin mutation detection performed on the SWGA enriched specimens matched the results obtained with a real-time PCR, indicating that all clinical specimens contained the A2058G mutation. SWGA-based enrichment also enabled sequencing of specimens within the range of detection limits for real-time PCR assays, suggesting that our NGS workflow can be adapted for *T. pallidum* detection in metagenomic samples.

In terms of expense, both methods are cost-effective for enriching *T. pallidum* genomic DNA, and while SWGA is cheaper than NEB+MDA, sequencing reagents are the true limiting factor for WGS. With the recent advancements in large-scale sequencing platforms, overall sequencing costs can be further reduced. While NovaSeq 6000 has a much higher potential for multiplex sequencing, our data shows compatibility of these enrichments for both NovaSeq 6000 and MiSeq platforms.

While we successfully enriched *T. pallidum* whole genomes in clinical specimens, the success of SWGA is limited by the constraint on primer size, which may reduce the selectivity for the target genome. Phi29 functions best between 30-35°C, and ramp-down incubations have been shown as an effective means of utilizing larger primers with increased melting temperatures (26-29). To help alleviate the constraints on primer size, we utilized a thermostable phi29 mutant which has a much higher optimal temperature at 45°C (30) compared to the 30-35°C functional range of the phi29 polymerase (26-27). This higher optimal temperature permits the use of longer oligonucleotides to be used in the SWGA reaction, potentially increasing the selectivity for the *T. pallidum* genome. The phi29 mutant has also shown to be more efficient, with a 3-hour exhaustion time when compared to the 8-16 hours required for the wild-type phi29 (30).

Our results show that SWGA is more sensitive, less cumbersome, and a faster method for enriching clinical specimens when compared to NEB+MDA, allowing for WGS of metagenomic samples with very low numbers of *T. pallidum*. In addition, the sequencing data generated is of sufficient quality to enable phylogenetic analyses and detection of mutations associated with azithromycin resistance. While the NEB+MDA was unsuitable for the clinical specimens in this study, our data suggests that it can be used for samples exceeding 129 genomic copies/µl.

Materials and Methods.

Specimen collection, *T. pallidum* strains used for WGS, and real-time qPCR. Specimens used in this study were collected from men presenting with lesions of primary or secondary syphilis to the Emory Infectious Diseases Clinic, Emory University Hospital Midtown (EUHM) in Atlanta, GA and St Louis County STD Clinic (STLC) in St. Louis, MO (Table 1). Patients were diagnosed with syphilis based on clinical presentation and serology testing. Fourteen swab specimens were collected in Aptima Multitest storage medium (Hologic, Inc., Marlborough, MA) at Emory Infectious Diseases Clinic and 1 specimen at St. Louis County STD Clinic (Table 1). All specimens were stored at -80°C until shipment on dry ice to the CDC. The *T. pallidum* Nichols reference strain was used for initial optimization and validation of the two enrichment methods. A recent rabbit propagated isolate, CDC-SF003, was also included for testing (Table 1; 7). Prior to study commencement, local IRB approvals were obtained from, Emory University, and St. Louis County Department of Public Health, and the project was approved at CDC

DNA was extracted from specimens and rabbit testis extracts using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD) following the manufacturer’s recommendations. Large-scale DNA extraction of three specimens was carried out on 1.5 ml of the Aptima stored specimen using the QIAamp DNA Mini Kit following the manufacturer’s recommendations for upscaling with slight modifications (Table 1). Proteinase K was added at 0.1X total sample volume, and AL Buffer and absolute ethanol were added at 1X total sample volume. Each sample was processed through a single column, washed following the manufacturer’s recommendations, and eluted in 100 µl AE Buffer (Qiagen). Following DNA extraction, each sample was tested by a real-time quantitative duplex PCR (qPCR) targeting the *polA* gene of *T. pallidum* and human RNase P gene (RNP) using a Rotor-Gene 6000 instrument (Qiagen) as previously described with modifications (7; see additional methods in supplemental materials).

Enrichment of *T. pallidum* by capture of CpG methylated host DNA and multiple displacement amplification (MDA). Initially, DNA concentration of extracts from clinical specimens and rabbit propagated strains were measured using the Qubit dsDNA HS assay (Thermo Fisher Scientific, Waltham, MA). Capture and removal of CpG methylated host DNA from samples were carried out using the NEBNext Microbiome DNA Enrichment Kit following the manufacturer's recommendations with modifications (New England Biolabs, Ipswich, MA). For all samples tested, 250 ng of DNA was subjected to two rounds of bead capture using the NEBNext Microbiome DNA Enrichment Kit and enriched treponemal genomic DNA was purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN). Enriched DNA samples were stored at -20°C until MDA was performed. MDA was carried out using the REPLI-g Single Cell Kit following the manufacturer's recommendations with slight modifications (Qiagen). Each MDA reaction was incubated at 30°C for 16 hr. Following amplification, the polymerase was inactivated at 65°C for 10 min, samples were purified with AMPure XP beads, and eluted with 100 µl 1X AE Buffer (Qiagen). For each enrichment using the REPLI-g Single Cell Kit, non-template controls were included to confirm the absence of *T. pallidum.*

A 10-fold dilution series on the Nichols strain was used to determine the limit of detection (LoD) for enrichment (see supplemental materials) with NEB+MDA followed by sequencing on an Illumina NovaSeq 6000. After DNA extraction, each dilution in the series was enriched by NEB+MDA, genomic copy numbers estimated by *polA* qPCR, and sequencing performed in triplicate. Enriched samples were diluted 1:10 prior to measuring RNP amplification. The LoD was set at the minimal genome copy number required to generate a ≥5X read depth with ≥95% genome coverage compared to the reference genome.

Selective whole genome amplification (SWGA) primer design, validation, and enrichment. Primers with an increased affinity to *T. pallidum* were identified using the *swga* Toolkit as previously described with slight modifications (https://www.github.com/eclarke/swga; 26; see supplemental materials). Eight primer sets (SWGA Pal 1-8), including 4 additional primer sets (SWGA Pal 9-12) generated by combining primers in the initial set (Table S1), were chosen for SWGA using the EquiPhi29 DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). To account for the 3’-5’ exonuclease activity of the phi29 polymerase, all SWGA primers were generated with phosphorothioate bonds between the last two nucleotides at the 3’ end (Table S1). Each of the 12 primer sets were tested in triplicate against the spiked sample diluted to an estimated 100 *T. pallidum polA* copies/µl (see supplemental materials).

Prior to SWGA enrichment, samples were denatured for 5 min at 95°C after adding 2.5 µl of DNA to 2.5 µl reaction buffer, containing custom primers, then placed immediately on ice until the Equiphi29 master mix, prepared as per manufacturer’s recommendations, was added (Thermo Fisher Scientific, Waltham, MA). MDA was carried out following the manufacturer’s recommendations with modifications (Thermo Fisher Scientific; 30). The reaction contained EquiPhi29 master mix, with EquiPhi29 Reaction Buffer at a final concentration of 1X, each primer at a final concentration of 4 µM, and nuclease-free H_2O was added to a final reaction volume of 20 µl. Reaction tubes were gently mixed by pulse vortexing and incubated at 45°C for 3 hr. MDA was stopped by inactivating the DNA polymerase at 65°C for 15 min. All reactions were purified using AMPure XP beads and eluted in 100 µl AE buffer (Qiagen). Non-template controls were included to confirm the absence of contaminate *T. pallidum* DNA.

Relative percent *T. pallidum* in each sample was calculated as shown in Figure S1. SWGA Pal 11 was chosen for testing the LoD for downstream genome sequencing post-SWGA enrichment using the 10-fold dilution series, excluding the undiluted (neat) spiked sample. All enriched samples were validated by *polA* real-time qPCR in triplicate.

Sequencing and genome analysis of *T. pallidum* strains. Libraries were prepared using the NEBNext Ultra DNA Library Preparation Kit for NovaSeq and NEBNext Ultra II FS DNA Library Preparation Kit for MiSeq sequencing following the manufacturer’s recommendations (New England Biolabs, Ipswich, MA). For the validation experiments, sequencing was carried out on the Nichols reference strain using the Illumina

NovaSeq 6000 platform following the manufacturer's recommendations (Illumina, San Diego, CA). Sequencing of isolate CDC-SF003 and swab specimens were carried out using the MiSeq v2 (500 cycle) platform following the manufacturer's recommendations (Illumina, San Diego, CA).

Post sequencing, reads were deduplicated, trimmed, and down selected for *T. pallidum* (supplemental materials). All down selected *T. pallidum* reads were mapped to the *T. pallidum* reference genomes, and *de novo* assembled. Phylogenetic analyses were performed as described in the supplemental materials. Apart from the genomes sequenced in this study, 122 high quality (with at least 5x read depth covering > 90% of the genome) *T. pallidum* genomes deposited in the NCBI's Sequencing Read Archive (SRA) under the BioProject number PRJEB20795 and PRJNA508872 were also included (12, 14). The publicly available raw sequencing data were re-analyzed to determine the quality as described in the supplemental materials. A second phylogenetic tree was also reconstructed by including all the genomes sequenced from the 10-fold dilution series for both NEB+MDA and SWGA enriched samples. Genomic sequencing data from samples included in the phylogenetic analyses covered at least 90% of the reference genome with 5X read depth. Variant calls for the A2058G and A2059G macrolide resistance mutations were validated using a real-time PCR assay as previously described (31).

Statistical analyses. Statistical analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria) using the R companion software RStudio (Rstudio, Boston, MA). Statistical significance was determined by analysis of variance (ANOVA) and Tukey post hoc multiple comparisons tests. *T. pallidum* percent DNA were normalized through Log_{10} conversions. Quantitative data are presented as means ± standard error. Differences were considered statistically significant if a $P < 0.05$.

Data availability. All sequencing data associated with this study were submitted to the National Center for Biotechnology Information's sequence read archive (SRA) under the BioProject accession ID PRJNA744275.

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Author Contributions

Allan Pillay and Ellen N. Kersh conceived the study. Allan Pillay, Charles M. Thurlow, Cheng Chen, and Lilia Ganova-Raeva designed the study. Charles M. Thurlow and Allan Pillay designed the enrichment protocols. Charles M. Thurlow designed the SWGA specific custom primer sets used during this study and performed all enrichment experiments. Charles M. Thurlow, Allan Pillay, Samantha S. Katz, Lara Pereira, Alyssa Debra, Kendra Vilfort, Yongcheng Sun, Kai-Hua Chi, and Damien Danavall performed the laboratory experiments and assisted with specimen collection. Kimberly Workowski, Stephanie E. Cohen, Hilary Reno, and Susan S. Philip collected clinical specimens and patient data. Mark Burroughs, Mili Sheth, and Charles M. Thurlow performed Illumina sequencing. Sandeep J. Joseph performed the bioinformatic analyses of the genomic data, phylogenetic analysis and contributed to the generation of tables and figures. Charles M. Thurlow and Sandeep J. Joseph performed data analysis. Charles M. Thurlow wrote and prepared the manuscript with oversight by Allan Pillay and contributions from Sandeep J. Joseph and Weiping Cao, which was reviewed by all authors for revisions.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. We declare that there are no competing interests.

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Tables

Table 1. Clinical and laboratory data for specimens and clinical isolate CDC-SF003.

Sample/ Isolate ID	Collection Year	Source	Gender	Sexual Status	Syphilis Stage	Site of Lesion	Antibody Titer (Assay)	qPCR (*T. pallidum polA* in DNA Extract)	Extraction Method	Reference
CDC-SF003	2017	San Francisco	Male	MSM	Primary	Penis	1:4 (VDRL)	9, 680 copies/µl	Standard	Pereira *et al.*, 2020
EUHM-001	2019	Atlanta	Male	MSM	Secondary	Neck	1:128 (RPR)	< 1 copy/µl	Standard	This study
EUHM-002	2019	Atlanta	Male	MSM	Secondary	Perianal	1:256 (RPR)	< 1 copy/µl	Standard	This study
EUHM-003	2019	Atlanta	Male	MSM	Secondary	Penis	1:32 (RPR)	< 1 copy/µl	Standard	This study
EUHM-004	2019	Atlanta	Male	MSM	Primary	Penis	1:4 (RPR)	106.7 ± 6.5 copies/µl	Standard	This study
EUHM-005	2019	Atlanta	Male	MSM	Secondary	Penis	1:64 (RPR)	< 1 copy/µl	Standard	This study
EUHM-006	2019	Atlanta	Male	MSM	Primary	Penis	1:16 (RPR)	< 1 copy/µl	Standard	This study
EUHM-007	2019	Atlanta	Male	MSM	Secondary	Hand	1:64 (RPR)	< 1 copy/µl	Standard	This study
EUHM-008	2019	Atlanta	Male	MSM	Secondary	Scrotum	1:64 (RPR)	0.9 ± 0.1 copy/µl	Standard	This study
EUHM-009	2019	Atlanta	Male	MSM	Secondary	Scrotum	1:64 (RPR)	< 1 copy/µl	Standard	This study
EUHM-010	2019	Atlanta	Male	MSM	Secondary	Scrotum	1:128 (RPR)	< 1 copy/µl	Standard	This study
EUHM-011	2019	Atlanta	Male	MSM	Primary	Penis	1:32 (RPR)	< 1 copy/µl	Standard	This study
EUHM-012	2019	Atlanta	Male	MSM	Primary	Penis	1:8 (RPR)	31.5 ± 0.5 copies/µl	Large Scale	This study
EUHM-013	2020	Atlanta	Male	MSM	Secondary	Penis	1:64 (RPR)	122 ± 1.2 copies/µl	Large Scale	This study
EUHM-014	2020	Atlanta	Male	MSM	Secondary	NA*	1:16 (RPR)	103 ± 6.7 copies/µl	Large Scale	This study
STLC-001	2020	St. Louis	Male	MSW	Primary	Penis	NR** (RPR)	28.8 ± 3.1 copies/µl	Standard	This study

[*] Not available

[**] Non-reactive

Table 2. Sequencing percent coverage for the Nichols isolates, clinical isolate CDC-SF003, and clinical specimens across the *T. pallidum* reference genome.

Sample	Enrichment method*	Clonal complex	*T.pallidum polA* post enrichment genome copies/µl	Raw read pairs	Non-host read pairs	Total read pairs after QC	Read pairs classified as *T. pallidum*	Percent of total read pairs classified as *T. pallidum*	Mean read depth	Percent genome covered ≥1X	Percent genome covered ≥5X	Percent genome covered ≥10X
Nichols_CDC	non-enriched	Nichols-like	NA***	4,053,500	3,645,649	3,588,414	70,299	1.96	6.33	86.26	60.30	22.28
Nichols_CDC**	SWGA	Nichols-like	11,565,333 ± 1,294,672	3,701,303	3,692,932	3,648,044	3,414,111	93.59	751.17	98.39	98.24	98.16
CDC-SF003	NEB + MDA	SS14-like	2,394,930 ± 135,210	5,798,777	3,988,173	3,949,036	129,998	3.29	46.44	98.87	98.60	98.01
EUHM-004	SWGA	Nichols-like	6,367,089.5 ±240,811.5	6,102,826	4,440,618	4,280,401	1,403,645	32.79	370.39	96.99	95.13	92.67
EUHM-012	SWGA	Nichols-like	2,140,753 ± 28,192	10,350,274	5,870,287	5,716,082	2,793,693	48.87	639.86	96.34	93.98	91.89
EUHM-013	SWGA	SS14-like	5,159,716 ± 220,318.5	11,975,324	11,966,460	11,838,431	8,308,234	70.18	2,503.96	98.72	98.56	98.37
EUHM-014	SWGA	Nichols-like	2,573,508 ± 221,900.5	11,250,518	9,266,926	9,059,022	2,355,426	26.00	930.87	98.79	98.49	98.04
STLC-001	SWGA	SS14-like	7,420,534 ± 719,765	11,293,960	7,770,834	7,721,767	3,004,631	38.91	1,133.43	98.32	95.94	94.10

[*] All sequencing was performed using Illumina's MiSeq v2 (500 cycle) platform

[**] Enrichment performed on 1,000 copies/µl *T. pallidum polA* input

[***] Not available

Figures

Fig 1. *T. pallidum polA* copies/µl for the 10-fold dilution series spiked samples enriched by the NEBNext Microbiome Enrichment Kit with REPLIg Single Cell MDA (NEB+MDA) or SWGA. The input *T. pallidum polA* copies/µl for each dilution is displayed as Non-Enriched. The y-axis has been $\log_{10}$ scaled for depiction of the Non-Enriched dilution series. Error bars represent standard error among three replicate enriched *T. pallidum* samples.

Fig 2. Relative percent *T. pallidum* Nichols DNA for Non-Enriched, NEBNext Microbiome Enrichment Kit with REPLIg Single Cell MDA (NEB+MDA), and SWGA enriched samples. Percent *T. pallidum* DNA was calculated based on the input DNA concentration and *polA* copies/µl (Non-Enriched), and the DNA concentration and *polA* copies/µl for the Nichols -spiked samples post-enrichment (NEB+MDA or SWGA). The y-axis has been $\log_{10}$ scaled for depiction of the Non-Enriched dilution series. Error bars represent standard error among three replicate samples.

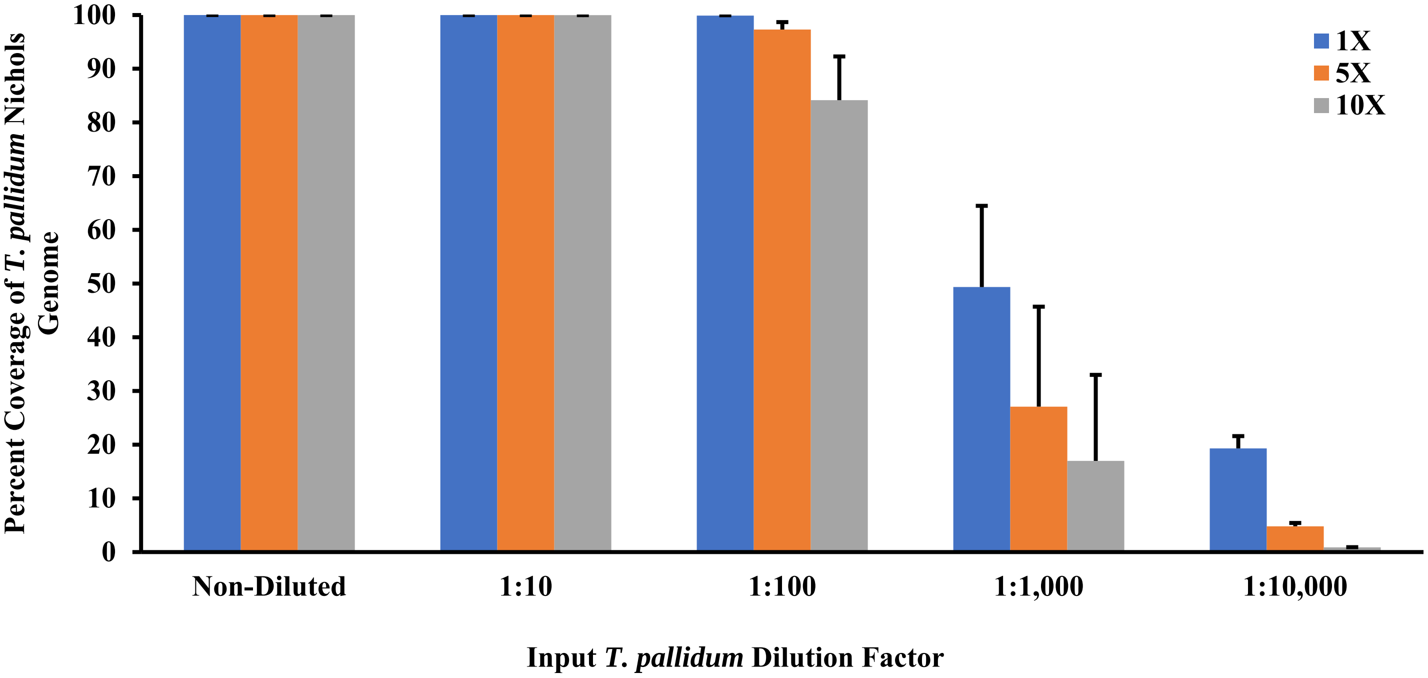
Fig 3. Percent coverage of sequencing reads of enriched *T. pallidum* Nichols spiked samples. (A) Sequencing reads of samples enriched using the NEB Microbiome Enrichment Kit and REPLIg Single Cell MDA (NEB+MDA). (B) Sequencing reads of samples enriched using SWGA. All samples were sequenced using the Illumina NovaSeq 6000 platform. Error bars represent standard error between the mapped reads derived from three replicate enriched Nichols samples.

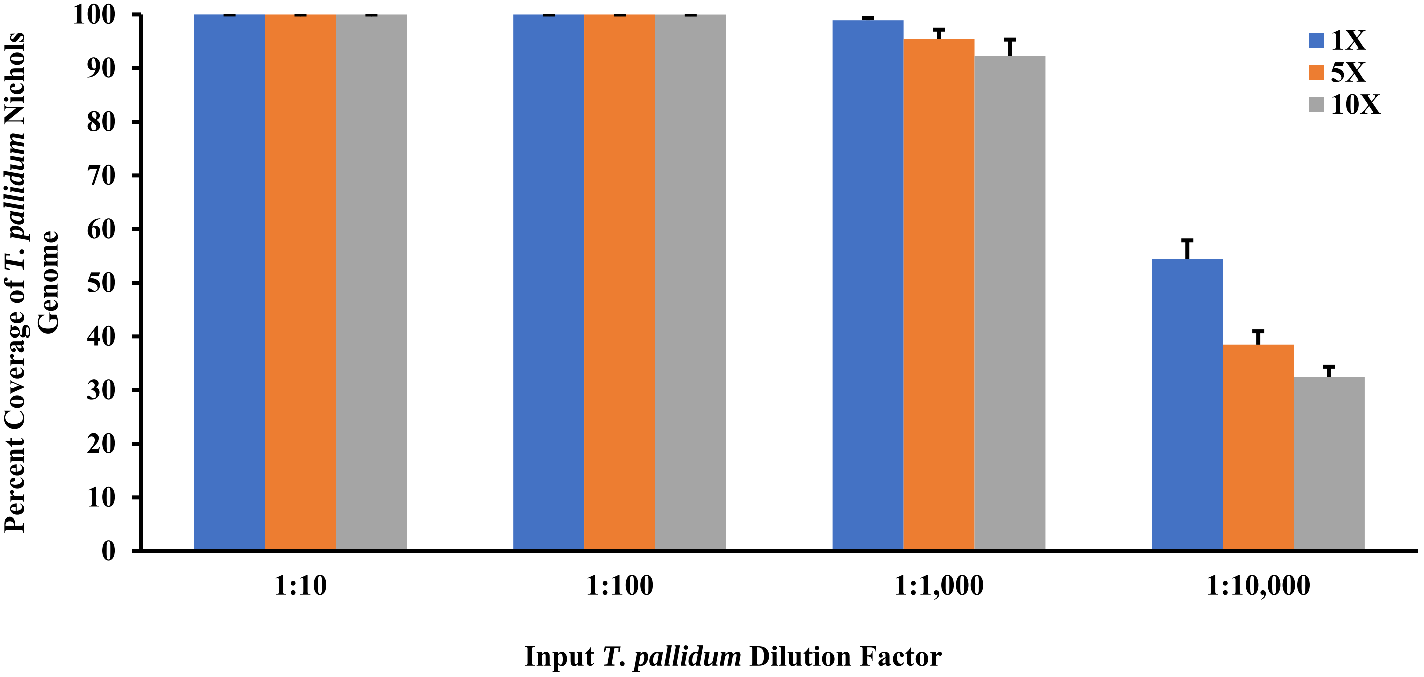
Fig 4. Percent coverage of isolates and clinical specimens. All samples were sequenced using the Illumina MiSeq v2 (500 cycle) platform. Percent of *T. pallidum* reads are derived from down selected *T. pallidum* reads. Prefiltered reads for Nichols-CDC were mapped to the Nichols reference genome (*NC_000919.1*). The prefiltered reads in all clinical isolates and specimens were mapped against the SS14 reference genome *(NC_021508.1*).

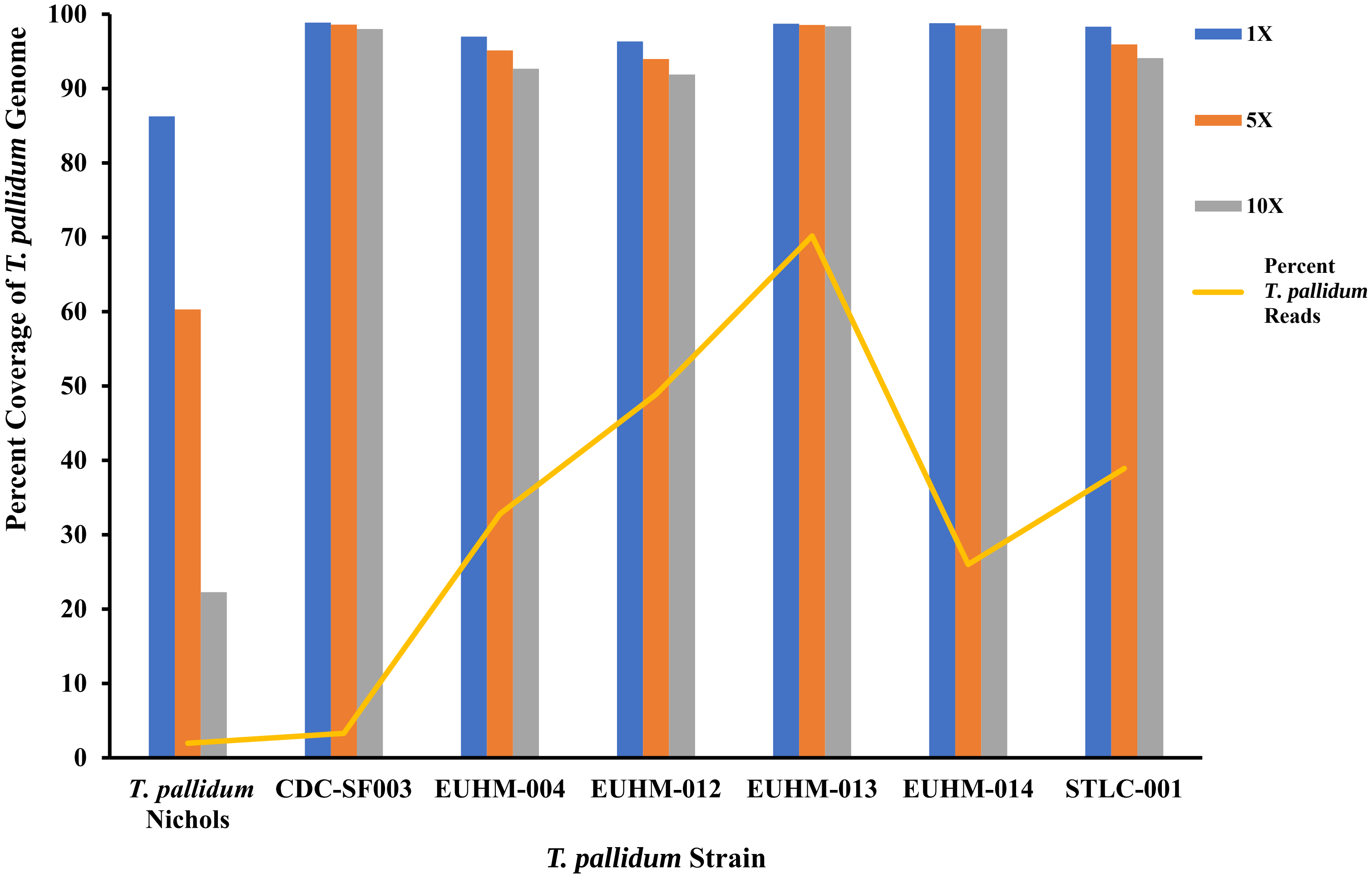
Fig 5. SWGA primer set validation. (A) *T. pallidum polA* copies/µl for the Nichols mock sample (1:100 diluted) enriched with each SWGA primer set. (B) Relative percent *T. pallidum* DNA for the Nichols

spiked sample (1:100 dilution) enriched with each SWGA primer set. Percent *T. pallidum* DNA was calculated based on the input DNA concentration and *polA* copies/µl for the Nichols mock samples post-SWGA enrichment. The y-axis has been $\log_{10}$ scaled for depiction of the relative percent *T. pallidum* post-enrichment with each primer set. Error bars represent standard error among three replicate Nichols samples.

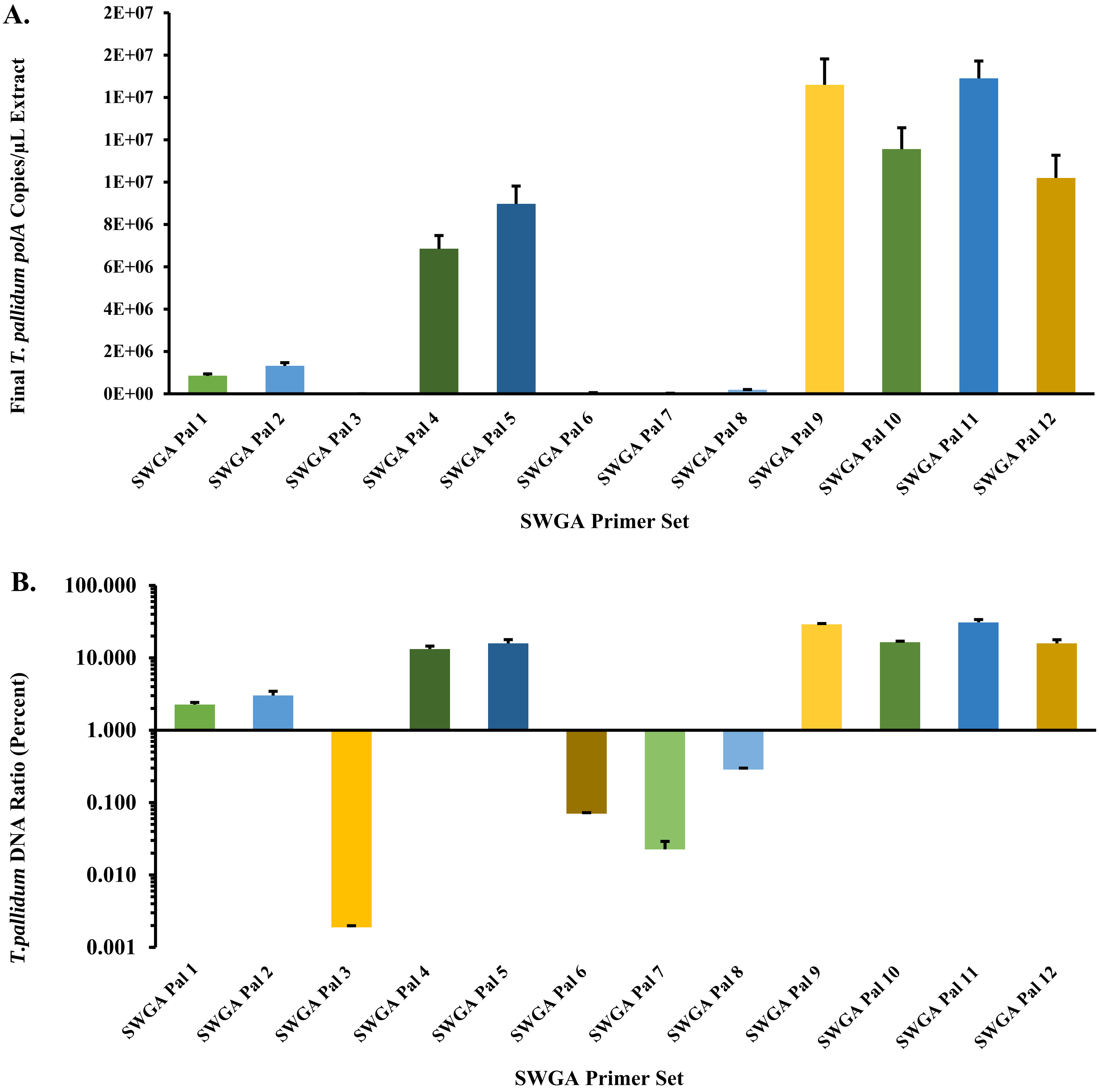
Fig 6. Maximum likelihood global phylogenetic tree of the clinical isolate/specimen genome sequenced in this study along with publicly available *T. pallidum* genomes. The two major lineages, Nichols-like and SS14-like are highlighted along with presence of genotypic mutation responsible for macrolide resistance and country of origin.

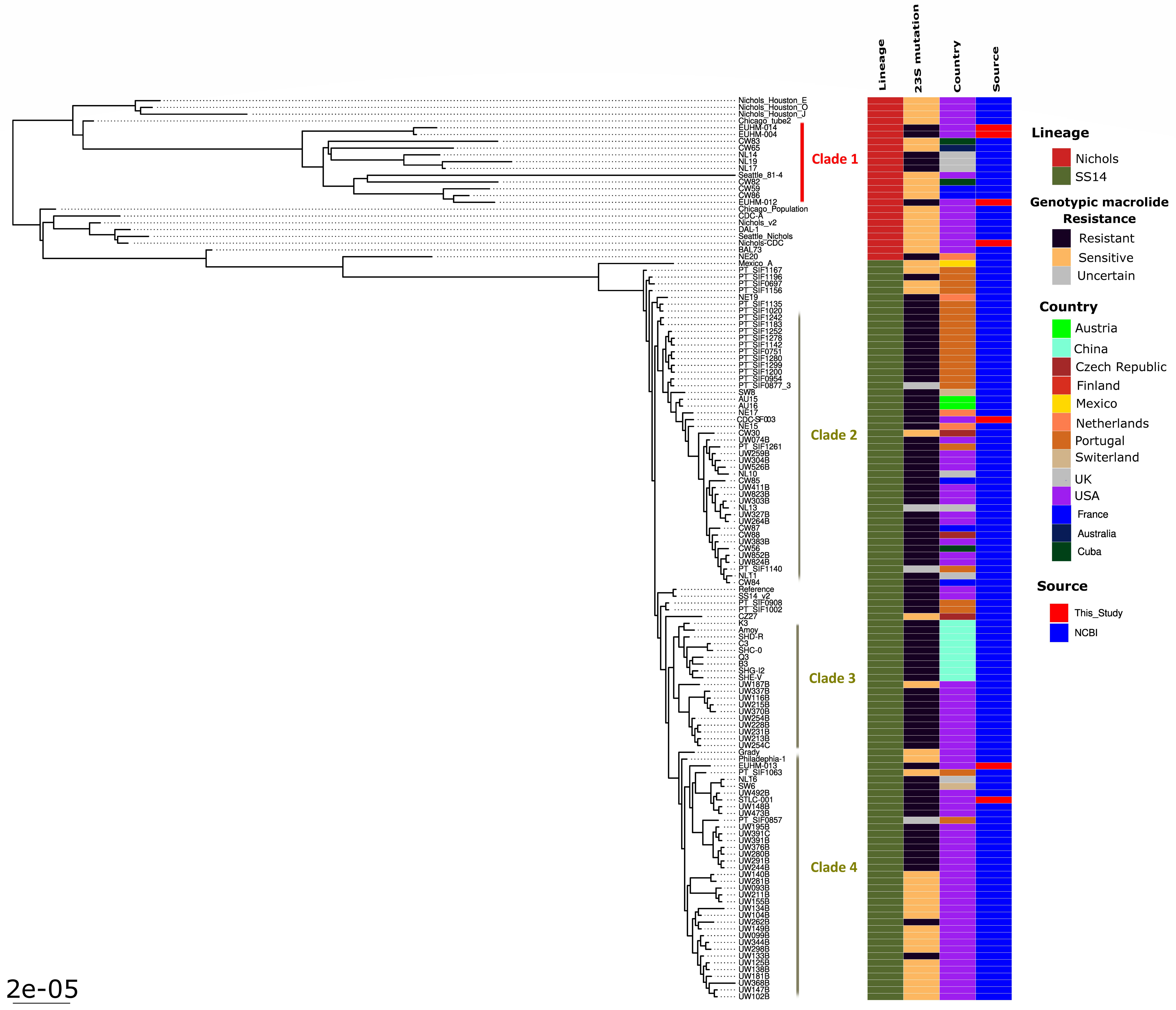

Percent Coverage of *T. pallidum* Nichols Genome
100
90
80
70
60
50
40
30
20
10
0
1X
5X
10X
Non-Diluted
1:10
1:100
1:1,000
1:10,000
Input *T. pallidum* Dilution Factor

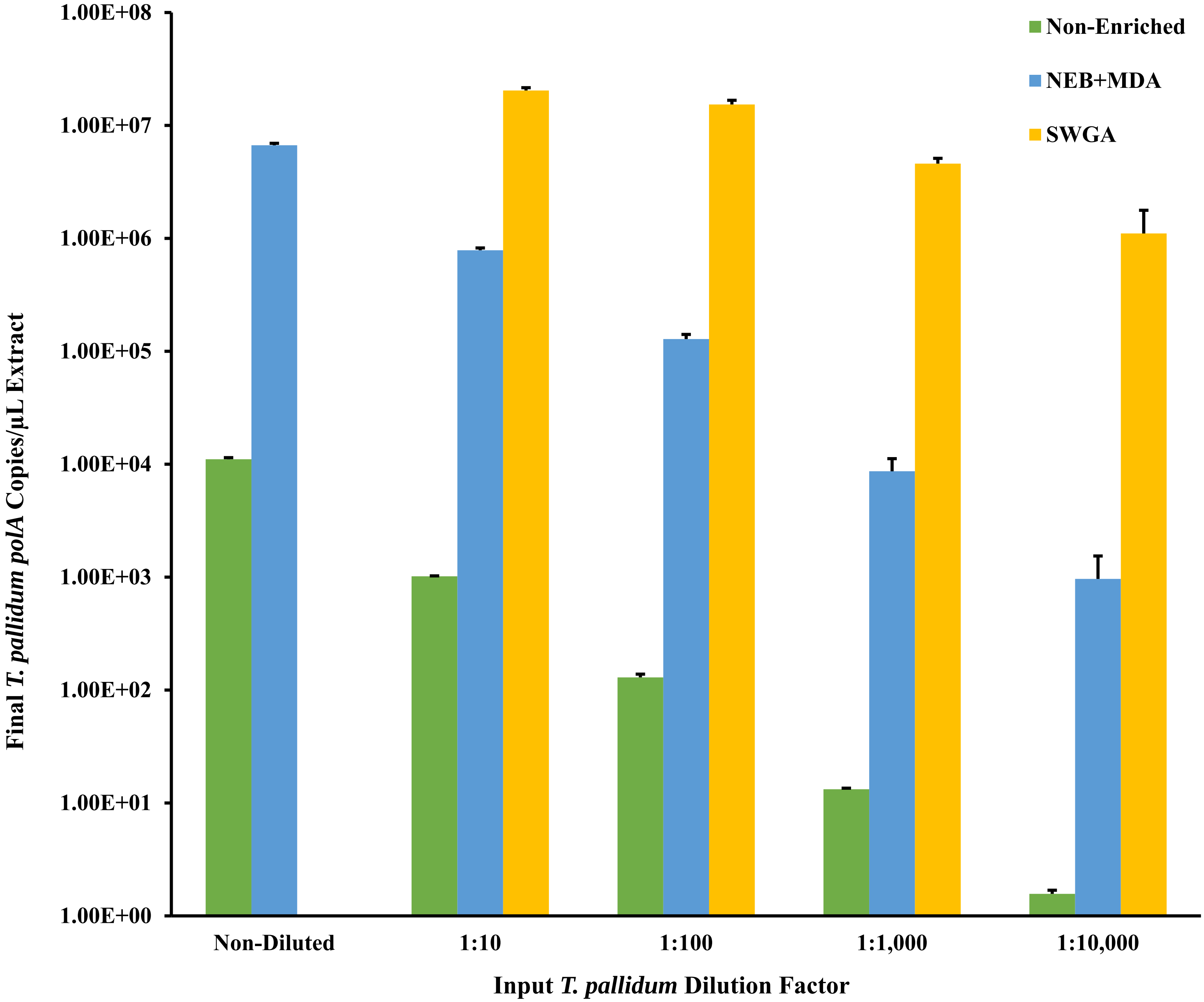

Percent Coverage of *T. pallidum* Nichols Genome
100
90
80
70
60
50
40
30
20
10
0
1X
5X
10X
1:10
1:100
1:1,000
1:10,000
Input *T. pallidum* Dilution Factor

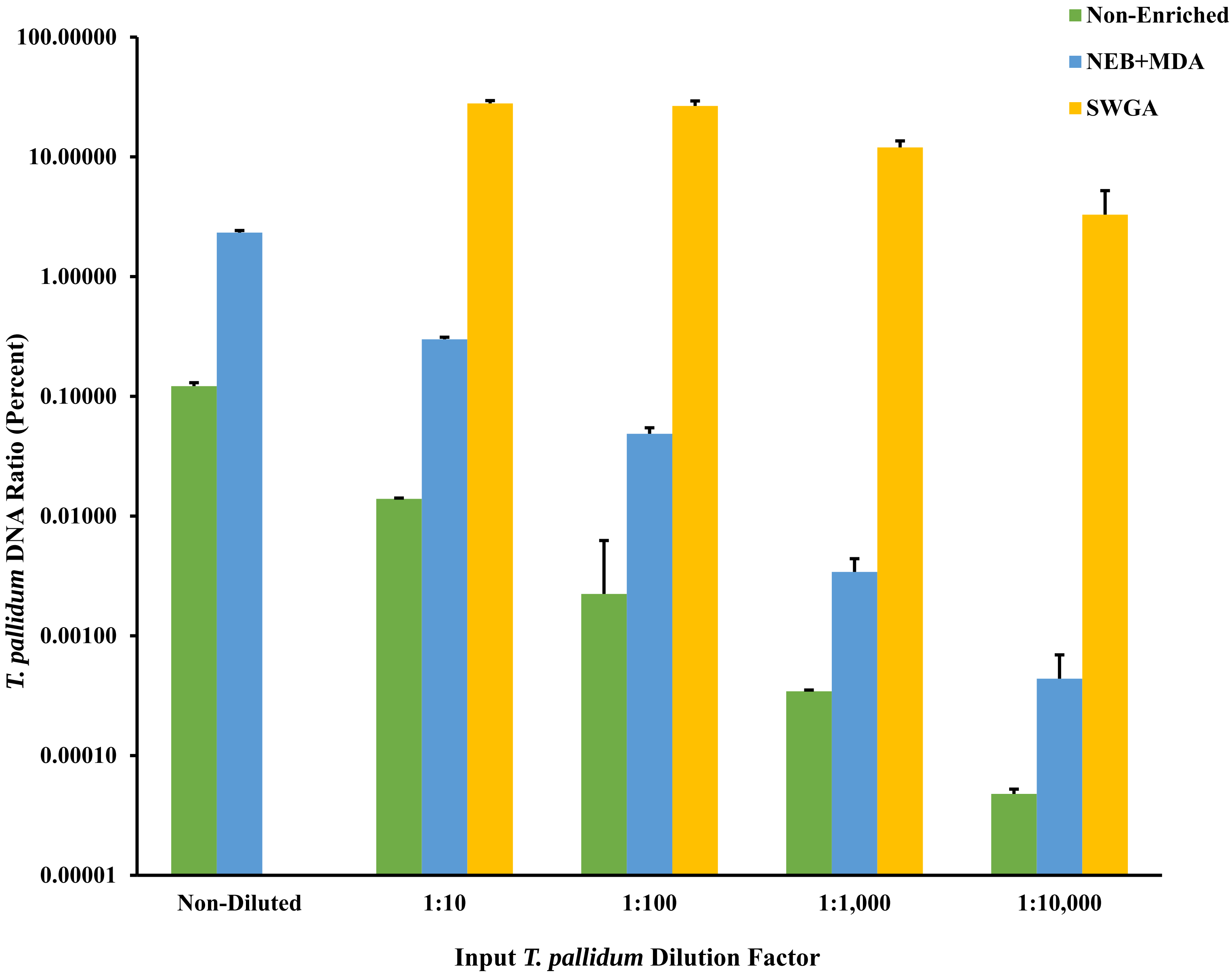


Percent Coverage of *T. pallidum* Genome
100
90
80
70
60
50
40
30
20
10
0
T. pallidum Nichols
CDC-SF003
EUHM-004
EUHM-012
EUHM-013
EUHM-014
STLC-001
T. pallidum Strain
1X
5X
10X
Percent *T. pallidum* Reads


A.
Final T. pallidum polA Copies/µL Extract
2E+07
2E+07
1E+07
1E+07
1E+07
8E+06
6E+06
4E+06
2E+06
0E+00
SWGA Pal 1
SWGA Pal 2
SWGA Pal 3
SWGA Pal 4
SWGA Pal 5
SWGA Pal 6
SWGA Pal 7
SWGA Pal 8
SWGA Pal 9
SWGA Pal 10
SWGA Pal 11
SWGA Pal 12
SWGA Primer Set
B.
T.pallidum DNA Ratio (Percent)
100.000
10.000
1.000
0.100
0.010
0.001
SWGA Pal 1
SWGA Pal 2
SWGA Pal 3
SWGA Pal 4
SWGA Pal 5
SWGA Pal 6
SWGA Pal 7
SWGA Pal 8
SWGA Pal 9
SWGA Pal 10
SWGA Pal 11
SWGA Pal 12
SWGA Primer Set


Lineage
23S mutation
Country
Source
Nichols_Houston_E
Nichols_Houston_O
Nichols_Houston_J
Chicago_tube2
EUHM-014
EUHM-004
CW83
CW65
NL14
NL19
NL17
Seattle_81-4
CW82
CW59
CW86
EUHM-012
Chicago_Population
CDC-A
Nichols_v2
DAL-1
Seattle_Nichols
Nichols-CDC
BAL73
NE20
Mexico_A
PT_SIF1167
PT_SIF1196
PT_SIF0697
PT_SIF1156
NE19
PT_SIF1135
PT_SIF1020
PT_SIF1242
PT_SIF1183
PT_SIF1252
PT_SIF1278
PT_SIF1142
PT_SIF0751
PT_SIF1280
PT_SIF1299
PT_SIF1200
PT_SIF0954
PT_SIF0877_3
SW8
AU15
AU16
NE17
CDC-SF003
NE15
CW30
UW074B
PT_SIF1261
UW259B
UW304B
UW526B
NL10
CW85
UW411B
UW823B
UW303B
NL13
UW327B
UW264B
CW87
CW88
UW383B
CW56
UW852B
UW824B
PT_SIF1140
NL11
CW84
Reference
SS14_v2
PT_SIF0908
PT_SIF1002
CZ27
K3
Amoy
SHD-R
C3
SHC-0
Q3
B3
SHG-I2
SHE-V
UW187B
UW337B
UW116B
UW215B
UW370B
UW254B
UW228B
UW231B
UW213B
UW254C
Grady
Philadephia-1
EUHM-013
PT_SIF1063
NL16
SW6
UW492B
STLC-001
UW148B
UW473B
PT_SIF0857
UW195B
UW391C
UW391B
UW376B
UW280B
UW291B
UW244B
UW140B
UW281B
UW093B
UW211B
UW155B
UW134B
UW104B
UW262B
UW149B
UW099B
UW344B
UW298B
UW133B
UW125B
UW138B
UW181B
UW368B
UW147B
UW102B
Clade 1
Clade 2
Clade 3
Clade 4
Lineage
Nichols
SS14
Genotypic macrolide Resistance
Resistant
Sensitive
Uncertain
Country
Austria
China
Czech Republic
Finland
Mexico
Netherlands
Portugal
Switerland
UK
USA
France
Australia
Cuba
Source
This_Study
NCBI
2e-05


1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Final *T. pallidum polA* Copies/µL Extract
Non-Enriched
NEB+MDA
SWGA
Non-Diluted
1:10
1:100
1:1,000
1:10,000
Input *T. pallidum* Dilution Factor



🟩 Non-Enriched
🟦 NEB+MDA
🟨 SWGA
100.00000
10.00000
1.00000
0.10000
0.01000
0.00100
0.00010
0.00001
T. pallidum DNA Ratio (Percent)
Non-Diluted
1:10
1:100
1:1,000
1:10,000
Input *T. pallidum* Dilution Factor