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Molecular Subtyping of *Treponema pallidum* from North and South Carolina

Victoria Pope,[1]* Kimberley Fox,[1,2]† Hsi Liu,[1] Anthony A. Marfin,[1]‡ Peter Leone,[2,3] Arlene C. Seña,[3] Johanna Chapin,[1] Martha B. Fears,[1] and Lauri Markowitz[1]

Division of STD Prevention, National Center for HIV, STD, and TB Prevention, Centers for Disease Control and Prevention, Atlanta, Georgia,[1] and Department of Health and Human Services, North Carolina,[2] and University of North Carolina,[3] Chapel Hill, North Carolina

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Patients from five clinics in North and South Carolina who had lesions suggestive of primary or secondary syphilis were evaluated using molecular techniques that allow the differentiation of *Treponema pallidum* strains on the basis of two variable genes, *tpr* and *arp*. Lesion samples were screened for the presence of *T. pallidum* DNA using PCR for *polA*, which represents a segment of the polymerase I gene that is unique to the spirochete. Twenty-seven of 154 lesion samples were found to contain *T. pallidum*, 23 of which had typeable DNA. Seven molecular subtypes were found (10f, 12f, 13f, 14f, 14g, 15f, and 16f); one to four subtypes were identified at each clinic. Subtype 14f was found in 52% of the typeable specimens and was distributed in four of the five clinics. Subtype 16f was found in 22% of specimens and was concentrated at one clinic. Further data are needed to define the role of this technique in examining the epidemiology of syphilis.

Syphilis is a sexually transmitted infection associated with an increased risk of acquisition and transmission of human immunodeficiency virus (HIV); infection in mothers can result in substantial congenital morbidity. Syphilis is endemic across the southeastern United States. In this region, several areas maintain rates that greatly exceed the Centers for Disease Control and Prevention's (CDC's) year 2000 objective of 4 cases of syphilis per 100,000 persons. In 2000, North Carolina had a rate of 18.6 cases per 100,000 and South Carolina had a rate of 21.4 per 100,000 for all stages of syphilis compared to a national rate of 11.2 per 100,000 (2).

Beginning in 1999, the CDC embarked on an effort to promote syphilis elimination as a national goal (1, 10). One facet of the plan was to monitor syphilis transmission in areas of endemicity and prevent outbreaks that result from dissemination of syphilis into areas that had not experienced recent transmission.

Prior to the development of a subtyping system, studying the epidemiologic relationships of syphilis in areas of endemicity and in focal outbreaks was not possible. Identification and culture of *Treponema pallidum* subsp. *pallidum*, the causative agent of syphilis, is complicated by the lack of a sustainable culturing system (3). Recent development at the CDC of a subtyping method using molecular techniques (9) may allow the creation of endemicity maps of "local" syphilis strains. This subtyping method for *T. pallidum* would allow the monitoring of changes in the prevalence and geographical distribution of strains over time and would determine which strains are introduced during outbreaks.

In North Carolina in the late 1990s, there was an increase in syphilis which appeared to spread from west to east across the central part of the state (http://www.cdc.gov/std/Syphilis/syphilis-stats-all-years.htm). This presented a unique opportunity to study the epidemiology of syphilis and to determine the interstrain variability of *T. pallidum* within selected regions of the Carolinas.

MATERIALS AND METHODS

Patient enrollment. Patients were enrolled in the study from five sexually transmitted disease clinics located in public health departments in North Carolina and South Carolina between November 1999 and January 2003. The durations of study participation by clinic differed due to requirements for approvals and other operational issues. Persons who had moist lesions (chancres, condyloma lata, or mucous patches) consistent with primary or secondary syphilis were eligible for enrollment. The protocol called for all routine clinical specimens to be collected prior to any study samples. Each clinic performed an initial diagnostic evaluation, which included dark-field microscopy or direct fluorescent antibody for *T. pallidum* (DFA-TP) and the rapid plasma reagin (RPR) tests. Swab specimens from lesions, slides of lesion exudates, and serum samples were then collected for research purposes and sent to the CDC, where the RPR, *T. pallidum* particle agglutination, and DFA-TP tests and PCR for *T. pallidum* (6) were performed. Slides used for dark-field microscopy were air dried before being sent to the CDC for DFA-TP. All enrollees received appropriate treatment according to the usual clinical practices during their clinic visits. Institutional Review Board approval was obtained from the University of North Carolina at Chapel Hill, the North Carolina Department of Health and Human Services, the South Carolina Department of Health and Environmental Control, and the CDC.

Laboratory methods. Lesion specimens were collected on swabs. Each swab was swirled vigorously in a tube of transport medium (AMPLICOR STD swab specimen collection and transport kit; Roche Diagnostic Systems, Branchburg, N.J.), pressed against the side of the tube to express excess liquid, and then discarded. The tube was capped and stored in a freezer at −20°C until it was shipped to the CDC on dry ice. The tubes were stored at −70°C until they were tested by PCR to detect the *polA* gene of *T. pallidum* (6). DNA was isolated from the ulcer exudate samples using a blood and tissue kit (QIAGEN, Valencia, Calif.) according to the manufacturer's instructions. The primers used, based on a 395-bp region of the *polA* gene that is unique to *T. pallidum*, were a 25-mer

* Corresponding author. Mailing address: Mail Stop A-12, OD/NCHSTP/CDC, 1600 Clifton Rd., NE, Atlanta, GA 30333. Phone: (404) 639-4583. Fax: (404) 639-3446. E-mail: vpope@cdc.gov.

† Current address: Global AIDS Program, National Center for HIV, STD, and TB Prevention, Centers for Disease Control and Prevention, Bangkok, Thailand.

‡ Current address: San Francisco Quarantine Station, Division of Global Migration and Quarantine, Centers for Disease Control and Prevention, San Francisco, California.

TABLE 1. Demographic characteristics of 154 participants

Characteristic	No. (%)[a]
Gender	
Female	49 (32)
Male	105 (68)
Age (yr; median = 31)	
≤19	9 (6)
20–29	63 (41)
30–39	40 (26)
40–49	28 (18)
≥50	14 (9)
Race/Ethnicity	
Black	116 (76)
Hispanic	10 (7)
Native American	12 (8)
White	14 (9)
Clinic site	
Durham County, NC	17 (11)
Guilford County, NC	5 (3)
Richland County, SC	2 (1)
Robeson County, NC	16 (10)
Wake County, NC	114 (74)

[a] Percentages may not equal 100 due to rounding.

forward primer (5′-CGTCTGGTCGATGTGCAAATGAGTG-3′) and a 26-mer reverse primer (5′-TGCACATGTACACTGAGTTGACTCGG-3′).

Samples that tested positive for the *polA* gene were subtyped using PCR, followed by gel electrophoresis to determine the number of acidic repeat protein gene (*arp*) repeats and restriction fragment length polymorphism to determine the banding pattern of the *T. pallidum* repeat protein gene (*tpr*) (9). The primers for amplification of the *arp* gene were a 20-mer forward primer (5′-CAAGTC AGGACGGACTGTCC-3′) and an 18-mer reverse primer (5′-GGTATCACCT GGGGATGC-3′) that were selected from unique sequences flanking the repeat region. The amplicon was electrophoresed on a 0.8% to 1.0% agarose gel to determine the number of repeats present in the *T. pallidum* strain.

A nested PCR was used to amplify the *tpr* gene. The first primer pair, derived from conserved regions in the *tpr* homologs, consisted of a 20-mer forward primer (5′-ACTGGCTCTGCCACACTTGA-3′) and a 20-mer reverse primer (5′-CTACCAGGAGAGGGTGAAGC-3′). The second primer pair, based on an internal region of the PCR product obtained from the first amplification of the *tpr* gene, was a 19-mer forward primer (5′-CAGGTTTTGCCGTTAAGC-3′) and a 20-mer reverse primer (5′-AATCAAGGGAGAATACCGTC-3′). The amplicon was then digested with the restriction endonuclease MseI. The digested product was electrophoresed on a 1.5% to 2.0% agarose gel to determine the digestion pattern (a through i), with the sizes and patterns analyzed using an ABI310 genetic analyzer (Applied Biosystems, Foster City, Calif.) (6, 8).

All serum samples were tested at the CDC using standard procedures for the RPR and the *T. pallidum* particle agglutination tests (5). Air-dried lesion exudate smears were examined using DFA-TP (5). The slides were fixed for 10 seconds in 100% methanol and then stained for 30 min using fluorescein isothiocyanate-labeled monoclonal antibody against the 37-kDa protein of *T. pallidum*.

TABLE 2. *polA* results by clinical diagnosis of genital lesion

Diagnosis	No. (%) *polA* positive[a]
Syphilis	
Primary	15/42 (36)
Secondary	9/12 (75)
Stage not given	3/7 (43)
Herpes	0/48
Other	0/32
Not specified	0/13

[a] Number positive by *polA*/number with clinical diagnosis.

TABLE 3. *polA* PCR results by dark-field microscopy and CDC DFA-TP results

polA status	No.[a]			
	Dark field N, DFA-TP N	Dark field N, DFA-TP P	Dark field P, DFA-TP N	Dark field P, DFA-TP P
Negative	105	1	7	1
Positive	8	5	1	7

[a] N, negative; P, positive.

RESULTS

A total of 154 persons from the five clinics were enrolled (Table 1). Sixty-eight percent of the participants were male; 67% were between 20 and 39 years of age. The Wake County clinic enrolled 74% of all the participants (Table 1) throughout the 38-month study period. Enrollment periods at the other sites varied from 2 months to 27 months. No enrolled cases were known sexual partners of other study participants or otherwise epidemiologically linked.

A total of 27 lesion specimens were positive by *polA* PCR, indicating the presence of *T. pallidum* DNA. All were from participants who had a clinical diagnosis of syphilis. Fifteen of the 27 specimens were from primary syphilis cases, 9 from secondary syphilis cases, and 3 from cases whose stage of syphilis was not provided (Table 2). *polA* was detected in a higher proportion of specimens from secondary syphilis cases than from primary syphilis cases (75% versus 36%).

Of the 27 *polA*-positive samples, 23 contained sufficient DNA for typing, including all 8 samples that were negative by dark-field microscopy and DFA-TP (Table 3). A total of seven subtypes were identified, with one to four subtypes at each clinic (Table 4). Subtype 14f was the most common, accounting for 52% of all typeable samples. Subtype 14f was found in all four North Carolina clinics and among primary and secondary syphilis cases (six and five cases, respectively). Four of the five 16f subtypes were found in one clinic, with one from a secondary syphilis case and four from primary syphilis cases. The 16f subtypes clustered around a 1-month time frame, with a 14f subtype appearing 4 months later in the same clinic (data not shown).

Results were available for dark-field microscopy, DFA-TP (CDC results), and *polA* testing from 135 participants (Table 3). CDC results for DFA-TP were used because there were only a small number of results available. There was agreement among all three tests for 112 participants (105 all negative; 7 all

TABLE 4. Distribution of *Treponema pallidum* subtypes by county

Clinic site	Subtype(s) (no.)[a]
Durham County	14f (1)
Guilford County	12f (1), 14f (1), 15f (1)
Richland County	16f (1)
Robeson County	14f (1), 16f (4)
Wake County	10f (2), 13f (1), 14f (9), 14g (1)

[a] Number of individuals with subtype.

positive). As mentioned above, eight patients had lesion specimens negative by dark-field microscopy and DFA-TP tests but positive on *polA* PCR. All but one of these patients had reactive RPR tests (titers from 1:8 to 1:256). Seven patients had lesion specimens that were positive only by dark-field microscopy.

DISCUSSION

In this study of *T. pallidum* subtypes in North and South Carolina, a total of seven subtypes were identified from patients with lesions consistent with primary or secondary syphilis. These subtypes included two *tpr* subtypes (f and g) and five *arp* subtypes (sizes 10, 12, 13, 14, and 16). Previous studies indicated that f and g subtypes differed by four bands while the *arp* subtypes differ by size (8). The most common subtype identified in our study was 14f. More than half of the typeable specimens were 14f, which was detected in participants from four of the five clinics. The only other large-scale study of *T. pallidum* subtypes conducted in the United States was done in Maricopa County, Arizona (11). The age and gender distributions of the participants in both studies were similar; however, the Maricopa study population was predominately Hispanic while our study population was predominately African American. In both studies, 14f was the predominant subtype. However, in the Carolinas, which represented a wider geographic area, fewer subtypes were identified (7 in the Carolinas; 10 in Maricopa). This may have been due to the larger number of isolates in Arizona. The greatest diversity in subtypes noted in our study occurred in Wake County, North Carolina, where the majority of the participants were enrolled.

Of note, subtype 16f appeared only during a short time frame and primarily at one clinic in Robeson County, which is located in the southeastern part of North Carolina along Interstate 95 near the South Carolina border. The only other 16f subtype was detected almost a year earlier at a clinic in Richland County, South Carolina, which is also on the Interstate 95 corridor. Although the numbers are small, this suggests a cluster of related syphilis cases that were unrelated to the other cases that presented in central North Carolina.

In other studies that included international sites (8, 9), subtypes 12f, 14f, and 16f were not identified outside the United States. Subtype 14g has been identified in Durban, South Africa (8), but not previously in the United States (7, 9). Subtypes 10f, 13f, and 15f have not been previously identified in the United States or international sites (7, 8, 9), while other subtypes not identified in our study have been reported from both African and U.S. sites. Of these, subtype 14d was most prevalent in Africa (9), and it has also been reported in the United States (Birmingham, Ala.; Chicago, Ill.; and Phoenix, Ariz.) (8).

The presence of HIV infection may influence the clinical presentation of syphilis. Because the known HIV prevalence in this study was low, and patients were not systematically tested, we cannot evaluate any relationship between HIV infection and the syphilis subtype or *polA* detection rate.

Since PCR can detect smaller numbers of organisms (6) than dark-field microscopy or DFA-TP, it is not surprising that some patients in our study had positive *polA* results with negative dark-field microscopy and DFA-TP test results. All of these patients' specimens had typeable *T. pallidum* DNA, and all but one had reactive RPR tests, suggesting that these results were true positives. Unfortunately, PCR for detection of *T. pallidum* is not commercially available, and the time required to conduct the test is not practical for use in sexually transmitted disease clinics, where it would be most useful.

There were two ulcer samples that were positive by DFA-TP but negative by PCR. A possible explanation for this finding is that the numbers of treponemes in the samples were low; furthermore, since the protocol called for collection of clinical samples prior to any study samples, there may have been too few spirochetes to be detected by PCR from the second swab. Another possibility is that the DFA-TP was falsely positive. The monoclonal antibody that was used in the DFA-TP is directed against the 37-kDa protein, which is a flagellar protein. In the original studies that were performed, this monoclonal antibody reacted only with *T. pallidum* subspecies (4). However, cross-reactivity with possible contaminating gastrointestinal spirochetes cannot be entirely ruled out.

One limitation of this study was that only 36% of the specimens from patients with a clinical diagnosis of primary syphilis were *polA* positive, which was a lower percentage than in a previous study (11). Factors that may be responsible for this include inadequate sample collection, collection of study samples after clinical samples, enrollment of patients with partially healed ulcers, possible self-medication with topical medications by the patients, and overdiagnosis of primary syphilis. The relatively low proportion of specimens with detectable *polA* may have skewed the measured distribution of subtypes in the population. Another limitation is that we may not have enrolled all the patients with primary or secondary syphilis who presented to the clinics during the study period, which affected the number of specimen subtypes that were identified in this study.

The data presented here suggest that *T. pallidum* subtyping based on variability in the *arp* and *tpr* genes may be a useful epidemiologic tool. In this study, we did not have a sufficient number of syphilis cases to determine local trends or movement of strains, and no patients representing epidemiologically linked cases were enrolled. Studies carried out over a longer time frame need to be performed, with more-vigorous attempts to obtain adequate lesion samples from patients with primary or secondary syphilis. Results from prior investigations (7, 11) indicate that whole blood could also be used to determine *T. pallidum* subtypes, which may be a more efficient way to obtain samples from epidemiologically linked cases. The Maricopa study and our Carolinas study, which were conducted in different parts of the country, suggest that subtype 14f may be the predominant strain circulating in the United States. However, additional investigations involving molecular differentiation of *T. pallidum* strains from other parts of the country are needed for further elucidation.

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