Molecular Differentiation of *Treponema pallidum* Subspecies

Arturo Centurion-Lara,^{1,2*} Barbara J. Molini,¹ Charmie Godornes,¹ Eileen Sun,^{2†}
 Karin Hevner,¹ Wesley C. Van Voorhis,^{1,2} and Sheila A. Lukehart^{1,2}

Departments of Medicine¹ and Pathobiology,² University of Washington, Seattle, Washington 98195

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***Treponema pallidum* includes three subspecies of antigenically highly related treponemes. These organisms cause clinically distinct diseases and cannot be distinguished by any existing test. In this report, genetic signatures are identified in two *tpr* genes which, in combination with the previously published signature in the 5' flanking region of the *tpp15* gene, can differentiate the *T. pallidum* subspecies, as well as a simian treponeme.**

The human pathogenic treponemes include three subspecies of *Treponema pallidum* (i.e., subsp. *pallidum* [syphilis], subsp. *endemicum* [bejel], and subsp. *pertenue* [yaws]) and *T. carateum* (pinta). This classification is based upon distinctive host ranges and clinical manifestations of each infection and, in part, saturation reassociation kinetic studies of the causative agents of syphilis and yaws (28). The World Health Organization estimates that there are 12 million new cases of syphilis per year (17), and the total number of cases of yaws, bejel, and pinta (the endemic treponematoses) worldwide is approximately 2.5 million (38), although good surveillance data are not available (1, 2, 5, 7, 21, 24, 32, 39). These treponemes cannot be cultured continuously in vitro and may cause life-long infections in untreated individuals. Classically, *T. pallidum* infections are characterized by episodes of active clinical disease interrupted by periods of asymptomatic latent infection.

The natural history of primate infection with the Fribourg-Blanc treponeme is unknown. This treponeme was isolated in 1962 from a baboon in Guinea (12, 13). It affects various species of primates, and its geographical distribution overlaps with regions where yaws is endemic in Western Africa (13). In 1971, Smith et al. showed that the Fribourg-Blanc treponeme is able to experimentally infect humans and to cause active infection (34).

The *T. pallidum* subspecies and the Fribourg-Blanc treponeme are morphologically indistinguishable by immunofluorescence or electron microscopy (9, 14, 19, 31) and induce similar histopathological changes and cross-reactive antibodies, indicating a high level of antigenic relatedness (3, 4, 10, 12, 14, 27, 30, 33, 34, 36, 37). We have earlier reported a single-base-pair change in the 5'-flanking region of the *tpp15* gene (6), which introduces an Eco47III restriction site and permits the differentiation of *T. pallidum* subsp. *pallidum* from the other subspecies by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified products. This single-nucleotide change distinguishes only syphilis from nonsyphilis treponemes but cannot differentiate subspecies *pertenue* from subspecies *endemicum*. We now report the identification of subspecies-unique genetic signatures in two members of the *tpr*

gene family: *tprC* and *tprI* (11). RFLP analysis of *tprC* and *tprI* amplicons, in combination with the Eco47III digestion of *tpp15* gene flanking region amplicons, permits the differentiation of each of the three *T. pallidum* subspecies and the unclassified simian isolate.

All treponemal isolates used in the present study, except for the Pariaman and Ghana 051 *pertenue* strains, were propagated in New Zealand White rabbits (25) with the approval of the University of Washington Institutional Animal Care and Use Committee. DNA for the Pariaman and Ghana 051 *T. pallidum* subsp. *pertenue* strains was generously provided by

TABLE 1. *Treponema* isolates

Strain	Origin	Yr isolated	Reference(s)
<i>T. pallidum</i> subsp. <i>pallidum</i> (venereal syphilis)			
Nichols ^a	Washington, D.C.	1912	29
Sea 81-4 ^b	Seattle, Wash.	1980	26
Chicago ^c	Chicago, Ill.	1951	37
Sea 81-3 ^b	Seattle, Wash.	1981	26
Mexico A ^c	Mexico	1953	37
<i>T. pallidum</i> subsp. <i>pertenue</i> (yaws)			
Gauthier ^d	Brazzaville	1960	15, 16
Samoa D ^c	Western Samoa	1953	37
Samoa F ^c	Western Samoa	1953	37
Pariaman ^e	West Sumatra	1988	9
CDC-1 ^f	Densuso, Ghana	1980	23
CDC-2 ^f	Akorabo, Ghana	1980	23
Ghana 051 ^e	Ghana	1988	8
<i>T. pallidum</i> subsp. <i>endemicum</i> (bejel)			
Bosnia A ^c	Bosnia	1950	37
Iraq B ^c	Iraq	1951	37
Unknown species (simian)			
Fribourg-Blanc ^c	Guinea	1966	13

^a Originally provided by James N. Miller, University of California, Los Angeles.

^b Strain isolated in Seattle by Sheila A. Lukehart, University of Washington, Seattle.

^c Strains provided by Paul Hardy and Ellen Nell, Johns Hopkins University, Baltimore, MD.

^d Provided by Peter Perine, Centers for Diseases Control and Prevention, Atlanta, GA.

^e DNA provided by Leo Schouls, National Institute for Public Health and Environment, Bilthoven, and Gerda Noordhoek, Public Health Laboratory, Friesland, The Netherlands.

^f Strains provided by Robert George and Victoria Pope, Centers for Disease Control and Prevention, Atlanta, GA.

* Corresponding author. Mailing address: Harborview Medical Center, Box 359779, 325 9th Ave, Seattle, WA 98104. Phone: (206) 341-5364. Fax: (206) 341-5363. E-mail: acentur@u.washington.edu.

† Present address: Rosetta Inpharmatics, Seattle, Wash.

FIG. 1. (A) RFLP analysis of the 493-bp *tprI* amplicons using BsrDI. Restriction fragments of 334 and 159 bp are seen only in *T. pallidum* subsp. *endemicum* strains. See also Table 2. (B) Simultaneous RFLP analysis of the 707-bp *tprC* amplicons using BsrDI and BsiEI. A BsrDI restriction site is found in the *T. pallidum* subsp. *endemicum* (Bosnia A and Iraq B) isolates, the Fribourg-Blanc isolate, and in the *T. pallidum* subsp. *pallidum* Nichols, Chicago, and Sea 81-4 isolates, yielding fragments of 547 and 160 bp. The BsiEI restriction site (572- and 135-bp fragments) is present in the *T. pallidum* subsp. *pallidum* Sea 81-3 and Mexico A strains which lack the BsrDI restriction site and differentiates them from the *T. pallidum* subsp. *endemicum* isolates. The *T. pallidum* subsp. *pertenu* amplicons remain uncut (707 bp). See also Table 2.

Leo Schouls and Gerda Noordhoek. The origin of the isolates or DNA is indicated in Table 1. No isolates of *T. carateum* are known to exist; therefore, we could not analyze this species. DNA from each treponemal strain was extracted as described elsewhere (6).

Full-length *tprC* and *tprI* open reading frames from the simian isolate and at least one strain of each *T. pallidum* subspecies, as well as regions of the *tprC* and *tprI* genes from 19 strains

(Table 1), were amplified, cloned, and sequenced as previously described (35). PCR primers for *tprC* (sense, 5'-TGAACGCGCTCAACATAGAC; antisense, 5'-CCCTCATCCGAGACAAAT) were used to generate an amplicon of 707 bp. This PCR product was then digested using the enzymes BsrDI (8 U) and BsiEI (10 U) in a 50- μ l reaction volume at 65 C for 16 h as described by the manufacturer (New England Biolabs, Beverly, MA). The restriction products were then loaded onto a 1%

agarose–1.5% NuSieve agarose (Cambrex, Rockland, ME) gel and visualized using ethidium bromide staining. Another set of primers (sense, 5'-AAGCACGCGTGATACATCC; antisense, 5'-ATCCCTCGCCTGTAAACTGA) was used to amplify a 493-bp fragment of *tpiI*. BsrDI digestion of 10 µl of this PCR amplicon was performed according to the manufacturer's instructions (New England Biolabs, Beverly, MA); the digestion products were also separated in 1% agarose–1.5% NuSieve agarose (Cambrex) gels.

All treponemal strains were also analyzed using the previously published method (6) for RFLP analysis of the 5' flanking region of the *tpi15* gene with redesigned primers (sense, 5'-TCCGCCTTCTCGCGTTTCTCG; antisense, 5'-TGCGAGGGTAGTTGCTGCTTCG). PCR was carried out as already described (9), and amplicons were digested as previously described. Each 50-µl digestion reaction contained either 25 U of AfeI (an Eco47III isoschizomer; NEB) per reaction.

Comparative analysis of the *tpiI* sequences in the three *T. pallidum* subspecies and the Fribourg-Blanc treponeme revealed a genetic signature region at positions 1740 to 1766 containing a BsrDI restriction site (NN/CATTGC) in *T. pallidum* subsp. *endemicum* (Fig. 1A). As predicted, amplicons from the two *T. pallidum* subsp. *endemicum* strains (Bosnia A and Iraq B) were cut at this unique site, yielding fragments of 334 and 159 bp, whereas the amplicons from *T. pallidum* subsp. *pertenue* and *T. pallidum* subsp. *pallidum* strains and the simian strain show a single uncut band at 493 bp (Fig. 1A and Table 2). In this analysis, the Fribourg-Blanc strain grouped with the *T. pallidum* subsp. *pertenue* strains.

In contrast to *tpiI*, the *tpiC* locus contains the signature GGTATTACTC at positions 1726 to 1735 in *T. pallidum* subsp. *pertenue* and some *T. pallidum* subsp. *pallidum* isolates (Fig. 1B). This signature differs from the corresponding regions in the *tpiC* genes of the *T. pallidum* subsp. *endemicum* strains and the remaining *T. pallidum* subsp. *pallidum* strains, which contain the BsrDI restriction site NN/CATTGC. Downstream of this first distinguishing region, there is a second signature that contains a BsiEI restriction site (CGRY/CG) unique to the subset of syphilis treponemes that lack the BsrDI restriction site. Consequently, BsrDI distinguishes *T. pallidum* subsp. *endemicum* and some *T. pallidum* subsp. *pallidum* isolates from the *T. pallidum* subsp. *pertenue* strains, and BsiEI differentiates *T. pallidum* subsp. *pertenue* from those *T. pallidum* subsp. *pallidum* treponemes that lack the BsrDI site. As predicted by sequence analysis (Fig. 1B and Table 2), the *T. pallidum* subsp. *pertenue* (Samoa D, Samoa F, and Gauthier) amplicons remain uncut, the *T. pallidum* subsp. *endemicum* (Bosnia A and Iraq B) and some *T. pallidum* subsp. *pallidum* (Chicago, Nichols, and Sea 81-4) isolates yield two BsrDI fragments of 547 and 160 bp, while the other *T. pallidum* subsp. *pallidum* (Mexico A and Sea 81-3) isolates give two BsiEI restriction fragments of 572 and 135 bp (see Fig. 1B). The CDC-1, CDC-2, Pariaman, and Ghana 051 *T. pallidum* subsp. *pertenue* strains, as expected, remain uncut (707 bp) by BsrDI (Table 2). In contrast to the *tpiI* results, the Fribourg-Blanc simian isolate here shows the same restriction pattern as the *T. pallidum* subsp. *endemicum* strains; thus, the combination of *tpiC* and *tpiI* analyses permits the differentiation of the simian isolate from the human isolates.

Because standard serological methods cannot distinguish

TABLE 2. Restriction analysis for differentiation of *Treponema* species and subspecies

Treponeme	Strain	<i>tpi15</i> gene 5' region ^a (Eco47III)	<i>tpiI</i> ^b (BsrDI)	<i>tpiC</i> ^c (BsrDI/ BsiEI)
<i>T. pallidum</i> subsp. <i>pallidum</i>	Nichols	+	–	+
	Chicago	+	–	+
	Sea 81-4	+	–	+
	Mexico A	+	–	(+)
	Sea 81-3	+	–	(+)
<i>T. pallidum</i> subsp. <i>pertenue</i>	Gauthier	–	–	–
	Samoa D	–	–	–
	Samoa F	–	–	–
	CDC-1	–	–	–
	CDC-2	–	–	–
	Pariaman	–	–	–
<i>T. pallidum</i> subsp. <i>endemicum</i>	Bosnia A	–	+	+
	Iraq B	–	+	+
Simian isolate	Fribourg-Blanc	–	–	+

^a *tpi15* gene 5' flanking region RFLP analysis was performed as previously published (6).

^b *tpiI* analysis: +, amplicons were cut by BsrDI (fragments of 334 and 159 bp); –, no cut.

^c *tpiC* analysis: +, amplicons were cut by the BsrDI (fragments of 547 and 160 bp); (+), amplicons were cut by BsiEI (fragments of 572 and 135 bp); –, no cut.

the treponemal infections from each other, these findings are of particular importance for infected persons from areas where yaws and bejel are endemic. A long-standing controversy in the treponemal field has been the whether venereal syphilis and the endemic treponematoses are caused by the same etiological agents. Hudson (20) hypothesized that the organisms are identical and that changes in the climatic conditions result in different clinical manifestations. In contrast, the hypothesis of Hackett (18) that these organisms are genetically distinct, although very closely related, is supported by our molecular studies and by the lack of cross-immunity among subspecies (37).

The phylogenetic status of the Fribourg-Blanc simian isolate has not yet been resolved. Initial serological and cross-immunity studies suggested that it is more closely related to subspecies *pertenue* than to subspecies *pallidum* (33). Our molecular analysis suggests that it is distinct from the *pertenue*, *endemicum*, and *pallidum* subspecies and may in fact be representative of another group of pathogenic treponemes. The ability of this strain to cause experimental active infections in humans indicates that primates may serve as reservoirs for zoonotic treponemal infections or new emerging human pathogens.

The strains for each subspecies that were tested in this analysis include all of the non-*T. pallidum* subsp. *pallidum* isolates currently available to the authors, and these strains represent geographically distinct regions of origin and diverse isolation dates. Our findings strongly suggest that the genetic differences identified here are indeed subspecies-specific and chromosomally stable. For the *endemicum* subspecies, the number of strains analyzed is small. However, studies of a larger number of strains, if they become available, may confirm our findings. We have previously demonstrated that even single nucleotide polymorphisms are reliable targets for differentiation of *T. pallidum* subspecies (6).

In addition to its utility in the specific diagnosis of treponemal infections from biopsy or swab samples, this method may permit subspecies differentiation in ancient DNA (22) from skeletal remains for studying the migration and evolution of the treponemal subspecies during the centuries. Ongoing studies in our laboratories may lead to the development of serological methods for differentiation of the treponematoses. Although serological tests would have more practical applications, the new methods described in the present study may also prove to be very useful in diagnosis and research.

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