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Molecular Typing of *Treponema pallidum* Clinical Strains from Lisbon, Portugal▽

C. Florindo,[1] V. Reigado,[1] J. P. Gomes,[1] J. Azevedo,[2] I. Santo,[2] and M. J. Borrego[1]*

Department of Infectious Diseases, National Institute of Health, Av. Padre Cruz, Lisbon 1649-016, Portugal,[1] and STD Clinics, Lapa Health Centre, R. São Ciro, Lisbon 1200-831, Portugal[2]

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A molecular system was used to subtype Portuguese *Treponema pallidum* clinical strains isolated from both skin lesions and blood. The study with this system constitutes the first typing study in a European country. Three *T. pallidum* subtypes were found: subtypes 14a (50%), 14d (45.2%), and 14f (4.8%). Further studies are needed to better characterize the isolates involved in syphilis outbreaks.

Syphilis, caused by the spirochete *Treponema pallidum* subsp. *pallidum*, is a multistage disease with a wide spectrum of clinical manifestations (8). Of particular relevance to public health is the recognition that syphilis increases the risk of transmission and acquisition of the human immunodeficiency virus (6). Until the end of the 20th century, the in vitro noncultivable condition of *T. pallidum*, allied with the high GC content of major portions of its genome (5), prevented the success of any strategy for the typing of *T. pallidum* clinical isolates. In 1998, Pillay et al. (13) made a very important step toward obtaining an understanding of the molecular epidemiology of *T. pallidum* by developing the only existing method for the genotyping of this pathogen. The method is based on the intrastrain variability of the acidic repeat protein gene (*arp*) and the *Treponema pallidum* repeat gene (*tpr*). To date, the laboriousness and low sensitivity of this procedure have limited its application to only five published studies (11, 13–15, 17), focused mainly in two countries, South Africa and the United States. Consequently, there are only 288 typed *T. pallidum* strains worldwide (11, 13–15, 17), which is a strikingly small number, considering the predicted syphilis incidence rate (18). In Portugal, early and congenital syphilis require mandatory notification (4); nonetheless, as in the rest of Europe, there is a complete lack of awareness of the diversity of the circulating *T. pallidum* strains. Here we report the results of a pioneer study in which we used the recent subtyping system developed by Pillay et al. at the Centers for Disease Control and Prevention (13) in order to identify and differentiate Portuguese *T. pallidum* isolates.

Four hundred sixteen individuals (104 women and 312 men) suspected of having early syphilis (on the basis of clinical data and serology), most of whom were attending the major Portuguese sexually transmitted disease clinic (located at the Lapa Health Centre in Lisbon, Portugal), were evaluated for *T. pallidum* infection between 2004 and 2007. *T. pallidum* DNA was extracted from skin lesions or blood samples by using a QIAamp DNA mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. *T. pallidum* was detected in 86 specimens (35 primary and 7 secondary lesions and 44 blood samples) by using a commercially available real-time PCR assay (Sacace, Caserta, Italy) that targets the gene coding for a recognized *T. pallidum* surface antigen, the 39-kDa basic membrane protein (*bmp*; locus TP1016 relative to the sequence of *T. pallidum* strain Nichols, GenBank accession number NC_000919 [3, 5]). The results for all real-time PCR-positive specimens were further confirmed by the use of a conventional PCR that targets a different *T. pallidum* gene, the DNA polymerase I (*polA*; locus TP0105 relative to the sequence of *T. pallidum* strain Nichols, GenBank accession number NC_000919 [5, 9, 10]). The *polA* 377-bp product was amplified as described by Liu et al. (9). No dark-field microscopy was conducted with the skin lesion exudates.

For molecular typing, the variable region of 60-bp repeats within the *arp* gene and the *tprE*, *trpG*, and *trpJ* genes were amplified and analyzed as described previously (11, 13), but by using a modified protocol. Briefly, 3 U of Bio-X-Act DNA polymerase (Bioline, London, United Kingdom) was used for the *arp* and *tpr* PCR mixtures. For both PCRs, the termocycling profiles consisted of an initial denaturation step at 94°C for 5 min, followed by 40 cycles (for *arp*) or 35 cycles (for *tpr*) at 94°C for 30 s and 62°C (for *arp*) or 60°C (for *tpr*) for 30 s and a final elongation step at 70°C for 2 min. The final extension step consisted of 10 min at 70°C. The number of *arp* gene tandem repeats was estimated as described previously (11) by using the O'range 100-bp DNA ladder (Peqlab, Erlangen, Germany).

By combining the results for the *arp* repeats and the *tpr* restriction pattern, we were able to fully genotype 64.3% (27/42) of the *T. pallidum* skin lesion isolates (23 from patients with primary syphilis and 4 from patients with secondary syphilis) and 34.1% (15/44) of the *T. pallidum* blood sample isolates. This poor proficiency, which has also been described by other authors (14), is largely related to the low rate of success of the *arp* gene PCR assay. The lower efficiency observed for the blood specimens is similar to that previously reported by Sutton et al. (17) for this type of biological sample. In fact, the treponemal load was probably higher in skin lesions than in blood samples (despite the capacity of the bacteria to readily disseminate into the bloodstream [8, 16]), and PCR-inhibitory substances are more likely to be present in blood than in ulcer

* Corresponding author. Mailing address: Department of Infectious Diseases, Instituto Nacional de Saúde Dr. Ricardo Jorge, Av. Padre Cruz, Lisbon 1649-016, Portugal. Phone: 351 217519241. Fax: 351 217526400. E-mail: m.jose.borrego@insa.min-saude.pt.

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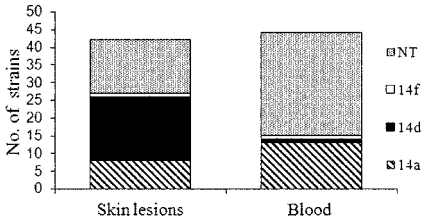

FIG. 1. Distribution of the *T. pallidum* subtypes among 86 patients with early syphilis (isolates were from skin lesions or blood). NT, nontypeable.

exudates (1). Despite these difficulties, the use of blood specimens seems to be a valuable tool for studies of the molecular epidemiology *T. pallidum* because it allows expansion of the typing of the organism to isolates recovered during the various stages of the disease. Moreover, it could shed light on the invasiveness of the different genotypes, which will contribute to a better understanding of the pathology of *T. pallidum*.

Overall, 48.8% (42/86) of the *T. pallidum*-positive specimens were typed (including six strains from three couples). This allowed the identification of subtypes 14a, 14d, and 14f (Fig. 1) and revealed a low level of genetic diversity. The predominant subtype, subtype 14a, was identified in 21 (50%) specimens, followed by subtypes 14d and 14f, which were found in 19 (45.2%) and 2 (4.8%) specimens, respectively. Subtypes 14a and 14d were also identified in other studies (11, 13, 14, 17), namely, in studies conducted in South Africa, where they were shown to be predominant in cerebrospinal fluid and genital ulcer specimens, respectively. To our knowledge, our work provides the first evidence of the existence of subtype 14f outside the United States, where it is the most common subtype (15, 17).

Curiously, the *T. pallidum* isolates from two of the three couples enrolled in the typing study presented discordant results by genotyping and were found to be of subtypes 14a and 14d. Possible reasons for this discrepancy (associated with *tpr* restriction pattern) may be related to (i) the existence of other sexual partners, (ii) the presence of a mixed infection, and (iii) genetic variation of the *tpr* gene subfamily II during host passage that results in different restriction patterns. This last hypothesis is supported by the findings of several other studies (2, 7, 12) that provided evidence of *tprK* sequence diversification during *T. pallidum* infection and host transmission. As recommended by Pillay et al. (14) and reinforced by our results, further studies will be necessary to evaluate the stability of the *T. pallidum tpr* subtypes within individual sexual networks.

Globally, genotyping of *T. pallidum* strains will contribute to the characterization of syphilis outbreaks, the evaluation of genotype invasiveness and virulence, differentiation between relapse and reinfection episodes, and knowledge about the distribution of bacterial types.

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