JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2009, p. 2510–2512 Vol. 47, No. 8
0095-1137/09/$08.00+0 doi:10.1128/JCM.00287-08
Copyright © 2009, American Society for Microbiology. All Rights Reserved.

Molecular Subtyping of *Treponema pallidum* subsp. *pallidum* in Lisbon, Portugal▽

R. Castro,[1] E. Prieto,[1] M. J. Águas,[2] M. J. Manata,[2] J. Botas,[2] and F. Martins Pereira[1]*

Instituto de Higiene e Medicina Tropical, Unidade de Doenças Sexualmente Transmitidas, Universidade Nova de Lisboa,[1] and Hospital Garcia de Orta,[2] Lisbon, Portugal

Received 12 February 2008/Returned for modification 20 April 2009/Accepted 13 May 2009

The objectives of this study were to evaluate the reproducibility of a molecular method for the subtyping of *Treponema pallidum* subsp. *pallidum* and to discriminate strains of this microorganism from strains from patients with syphilis. We studied 212 specimens from a total of 82 patients with different stages of syphilis (14 primary, 7 secondary and 61 latent syphilis). The specimens were distributed as follows: genital ulcers ($n = 9$), skin and mucosal lesions ($n = 7$), blood ($n = 82$), plasma ($n = 82$), and ear lobe scrapings ($n = 32$). The samples were assayed by a PCR technique to amplify a segment of the polymerase gene I (*polA*). Positive samples were typed on the basis of the analysis of two variable genes, *tpr* and *arp*. Sixty-two of the 90 samples positive for *polA* yielded typeable *Treponema pallidum* DNA. All skin lesions in which *T. pallidum* was identified (six of six [100%]) were found to contain enough DNA for typing of the organism. It was also possible to type DNA from 7/9 (77.7%) genital ulcer samples, 13/22 (59.1%) blood samples, 20/32 (62.5%) plasma samples, and 16/21 (76.2%) ear lobe scrapings. The same subtype was identified in all samples from the same patient. Five molecular subtypes (subtypes 10a, 14a, 14c, 14f, and 14g) were identified, with the most frequently found subtype being subtype 14a and the least frequently found subtype being subtype 10a. In conclusion, the subtyping technique used in this study seems to have good reproducibility. To our knowledge, subtype 10a was identified for the first time. Further studies are needed to explain the presence of this subtype in Portugal, namely, its relationship to the *Treponema pallidum* strains circulating in the African countries where Portuguese is spoken.

Syphilis is a sexually transmitted infection caused by *Treponema pallidum* subsp. *pallidum* (*T. pallidum*) and has a worldwide distribution, which remains important due to its strong association with the increased rates of acquisition and transmission of the human immunodeficiency virus (1, 3, 6, 7).

In Portugal and in accordance with the Portuguese General Direction of Health, there were 120 cases of recently acquired syphilis in 2006, which corresponds to an incidence rate of $1.20/10^5$ population, and 19 cases of congenital syphilis, which corresponds to an incidence rate of $0.13/10^5$ population, in the same year (2). However, when unpublished data from dermatology clinics in Portugal are taken into account (personal communications, 2002), syphilis is highly underreported.

Until some years ago, strains of *T. pallidum* could not be differentiated. Identification of the organism was complicated and there was no means of sustainable culture for this microorganism, which can be cultured only in experimental animals. This makes understanding of the pathogenesis and epidemiology of *T. pallidum* difficult. A technique that uses a combination of PCR amplification and restriction fragment length polymorphism (RFLP) analysis of two different gene targets (*arp* and *tpr*) was developed and used as a molecular typing system to differentiate between strains of *T. pallidum* (12). The number of 60-bp tandem repeats within the *arp* gene, indicated by a lowercase letter that designates the RFLP profile of a segment of the *tprE*, *trpG*, and *trpJ* genes, supports this typing system.

The capacity to differentiate strains of *Treponema pallidum* is important, since it makes it possible to know the diversity of circulating subtypes, to monitor changes in the prevalence and geographical distribution of the strains over time, and to determine which new strains have been introduced in a specific area.

The present study, based on the subtyping system referred to above, had the following objectives: to evaluate the reproducibility of the molecular subtyping method and to discriminate strains of *T. pallidum* from patients with syphilis from one area of Lisbon, Portugal.

MATERIALS AND METHODS

Between June 2003 and July 2005, patients attending a hospital and a sexually transmitted infection (STI) clinic in the Lisbon area were enrolled in the study if they had clinical manifestations and/or if the results of serological tests for syphilis (the rapid plasma reagin and fluorescent treponemal antibody tests) were reactive. Their stage of syphilis (primary, secondary, or latent syphilis) was classified according to their clinical symptoms and signs and the results of the serological tests.

The Scientific Council of the Instituto de Higiene e Medicina Tropical, which represents the committee that evaluates research with human subjects, approved the study.

Plasma was obtained from total blood with EDTA and was collected from every patient after informed consent was given. If a genital ulcer or a skin or mucosal lesion was present, the exudate was also obtained by using a Dacron-tipped swab. An ear lobe scraping was obtained from most of the patients, since, as postulated by A. W. Sturm (personal communication) and like *Borrelia* species, *T. pallidum* hides in capillary beds during the asymptomatic phase of the disease. To obtain this specimen, a scalpel was used to scrape the ear lobe and

* Corresponding author. Mailing address: Instituto de Higiene e Medicina Tropical, Rua da Junqueira, no. 96, Lisbon 1349-008, Portugal. Phone: 00351-213652600. Fax: 00351-213632105. E-mail: ritacastro@ihmt.unl.pt.

▽ Published ahead of print on 3 June 2009.

TABLE 1. Results of *polA* PCR by stage of syphilis and type of sample

Stage of syphilis	No. of samples positive by *polA* PCR/total no. of samples tested (%)			
	Blood	Plasma	Ear lobe scrapings	Lesion
Primary	4/14 (28.6)	6/14 (42.9)	4/5 (80)	9/9 (100)
Secondary	4/7 (57.1)	5/7 (71.4)	3/3 (100)	6/7 (85.7)
Latent	14/61 (23)	21/61 (34.4)	14/24 (58.3)	
Total	22/82 (26.8)	32/82 (39)	21/32 (65.6)	15/16 (93.8)

TABLE 2. Number of samples with organisms that could be typed by stage of syphilis and type of sample

Stage of syphilis	No. of samples with organism that could be typed/total no. of samples tested (%)			
	Blood	Plasma	Ear lobe scrapings	Lesion
Primary	3/4 (75)	5/6 (83.3)	4/4 (100)	7/9 (77.7)
Secondary	3/4 (75)	4/5 (80)	3/3 (100)	6/6 (100)
Latent	7/14 (50)	11/21 (52.4)	9/14 (64.3)	
Total	13/22 (59.1)	20/32 (62.5)	16/21 (76.2)	13/15 (86.7)

the resulting blood was collected with a sterile dry Dacron swab. After the scraping was collected, the swabs were vigorously agitated in a tube containing 1 ml of sterile phosphate-buffered saline for 30 s and pressed against the side of the tube to express the liquid. Afterwards, the swabs were discarded. These specimens were stored in a freezer (−4°C) for 1 or 2 days and were then sent to the Sexually Transmitted Disease Unit of the Instituto de Higiene e Medicina Tropical, where they were immediately studied.

On arrival at the laboratory, the samples were divided into two aliquots, one to be processed immediately and the other to be stored at −80°C for later use, if necessary. The blood was centrifuged to obtain the plasma and was divided into aliquots.

DNA from all specimens (blood, plasma, lesion exudates, and ear lobe exudate scrapings) was extracted with a QIAamp blood/tissue minikit (Qiagen), in accordance with the manufacturer's instructions, with the exception that for total blood with EDTA, 400 μl was used, whereas the manufacturer recommends that 200 μl be used. The incubation period used was also increased to 4 h instead of the recommended 10 min.

All samples were screened for *T. pallidum* DNA by a diagnostic PCR technique (*polA* PCR), described by Liu et al. (8), that amplifies a 378-bp region of the *polA* gene. Molecular subtyping was performed with specimens positive by this assay by using a PCR technique described by Pillay et al. (12), which is based on the following target genes: the acidic repeat protein (*arp*) and the *Treponema pallidum* repeat (*tprE*, *tprG*, *trpJ*) of the *tpr* gene family.

The primers for the amplification of the 60-bp tandem repeat region within the *arp* gene consisted of a 20-mer forward primer, primer ARP-1 (5′-CAA GTC AGG ACG GAC TGT CC-3′), and an 18-mer reverse primer, primer ARP-2 (5′-GGT ATC ACC TGG GGA TGC-3′), corresponding to the size of the amplified fragments in relation to the 60-bp tandem repeat region.

DNA amplification was performed in a reaction mixture with a total volume of 100 μl containing 10 μl of a 10× PCR buffer with 2 mM of $MgCl_2$ (Citomed), 5 μl deoxynucleoside triphosphates (10 mM each of dTTP, dCTP, dATP, and dGTP; Ultrapure dNTP set; Amersham Pharmacia Biotech), 100 pmol of each primer, 2 U of *Taq* polymerase (Imolase; Citomed), 10 μl of DNA extracted from each clinical specimen, and water to complete the volume to 100 μl. Five microliters of *Treponema pallidum* Nichols strain DNA and 10 μl of DNA extracted from the blood of individuals without any clinical history of syphilis and a nonreactive syphilis serology result were used as positive and negative controls, respectively. Distilled water instead of DNA was used as a control for PCR contamination.

The PCR was performed in a thermocycler (Master Cycler Personal; Eppendorf). The initial cycle was 95°C for 5 min to activate the polymerase. This initial cycle was followed by 40 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 2 min 30 s. The final extension consisted of one cycle at 72°C for 15 min, after which the tubes were stored at 4°C until they were analyzed. The amplicons were electrophoresed on a 2% agarose gel (Bio-Rad), together with a 100-bp DNA ladder (Citomed), at 100 V for 1 h. The number of *arp* gene tandem repeats from the samples studied was estimated by comparison with the molecular weight marker fragments and the *arp* PCR product from the Nichols strain of *T. pallidum* (14 repeats).

The *tpr* gene was amplified by a nested PCR (11). Primers consisting of a 20-mer forward primer, primer B1 (5′-ACTGGCTCTGCCACACTTGA-3′), and a 20-mer reverse primer, primer A2 (5′-CTACCAGGAGAGGGTGAAG C-3′), which amplify a fragment of 2,186 bp, were used for the first PCR, while for the second PCR, primers consisting of an 18-mer forward primer, primer IP6 (5′-CAG GTT TTG CCG TTA AGC-3′), and a 20-mer reverse primer, primer IP7 (5′-AATCAAGGGAGAATACCGTC-3′), which amplify an internal region of 1,836 bp of the amplicon obtained from the first amplification, were used. The mixture for the first PCR contained 10 μl of 10× PCR buffer with 2 mM of $MgCl_2$, 5 μl of the deoxynucleoside triphosphate mixture (10 mM each of dTTP, dCTP, dATP, and dGTP; Ultrapure dNTP set; Amersham Pharmacia Biotech), 60 pmol of each primer, 5 U of *Taq* polymerase (Imolase; Citomed), 10 μl of DNA extracted from the clinical specimen, and water to achieve a total reaction volume of 100 μl. The amplification conditions were 1 cycle of 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 59°C for 2 min, and 72°C for 2 min. The final extension was one cycle at 72°C for 15 min. Each product from this first amplification was submitted to a second amplification with the second pair of primers. The PCR mixture contained 6 μl of DNA template from the first *tpr* PCR, 10 μl of 10× reaction buffer containing 2.5 mM of $MgCl_2$ (Citomed), 5 μl deoxynucleoside triphosphates (10 mM each of dTTP, dCTP, dATP, and dGTP), 60 pmol of each primer, 5 U of *Taq* polymerase (Imolase; Citomed), and water to achieve a final volume of 100 μl. The following conditions were used for amplification: 1 cycle of 95°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension of 1 cycle at 72°C for 10 min. The Master Cycler Personal thermocycler (Eppendorf) was used for all PCR techniques.

To determine the variability of the *tpr* gene, the product obtained from the nested PCR was analyzed by RFLP with the restriction endonuclease MseI. The restriction mixture consisted of 10 μl of the PCR amplicon, 2 μl of 10× reaction buffer (Gibco/Life Techonologies), 5 U of restriction enzyme, and water to achieve a total volume of 20 μl; the mixture was incubated overnight at 37°C. The fragments obtained were separated by electrophoresis on a 2% agarose gel for analysis of the digestion pattern. This was obtained by comparing the sizes of the restriction fragments of the samples with the sizes of the fragments of the molecular weight marker and the fragments of the *tpr* amplicon of the Nichols strain of *T. pallidum*.

The subtype was determined by combining the results for the number of repeated sequences of 60 bp of the *arp* gene and the *tpr* RFLP patterns and is designated with a lowercase letter, as described by Pillay et al. (12).

RESULTS

A total of 82 patients with different stages of syphilis (14 with primary syphilis, 7 with secondary syphilis, and 61 with latent syphilis) were enrolled in the study. A total of 212 specimens were obtained from these patients.

The *polA* PCR technique identified *Treponema pallidum* DNA in 90/212 (42,5%) specimens (Table 1).

Among these positive samples, 62/90 (68.9%) had sufficient DNA for the organism to be subtyped (Table 2). The organisms from all skin lesions (six of six [100%]) positive by the *polA* PCR were subtyped. It was also possible to subtype the *T. pallidum* organisms by use of the DNA obtained from 7/9 (77.7%) genital ulcer specimens, 13/22 (59.1%) blood specimens, 20/32 (62.5%) plasma samples, and 16/21 (76.2%) ear lobe scrapings.

The same subtype was found in every sample from the same patient.

A total of five subtypes were identified: subtypes 10a, 14a, 14c, 14f, and 14g. The subtype found the most frequently was subtype 14a, which was found in 65% (13/20) of the patients from whom enough DNA was recovered for subtyping, fol-

lowed by subtypes 14f, 14c, 14g, and 10a, which were found in 40% (8/20), 25% (5/20), 20% (4/20), and 10% (2/20) of the patients, respectively.

DISCUSSION

In recent years, molecular biology techniques have become available and have been applied to evaluation of the organisms responsible for STIs, making it possible to subtype the different etiological agents of STIs (4, 9, 10, 12, 15).

Although *T. pallidum* was identified a century ago, until recently, strains of this organism could not be differentiated. In the study described here, we have used the typing system developed by Pillay et al. (12) to evaluate the molecular subtypes of *T. pallidum* circulating in the Lisbon area.

We have tried to subtype the *T. pallidum* DNA from patients with different stages of syphilis from whom different types of samples were collected.

Very few studies have been performed for *T. pallidum* subtyping. In most of them, only genital ulcers or skin lesions were studied (12–14), with the exception of the studies of Sutton et al. (16) and Molepo et al. (10), who subtyped *T. pallidum* DNA from 4/15 (26.6%) blood samples and 13/28 (46.4%) cerebrospinal fluid samples, respectively. In Portugal, Florindo et al. (4) managed to type the *T. pallidum* DNA from 42/86 (48.8%) lesions and blood specimens. However, ear lobe scrapings and plasma samples were used for subtyping for the first time in the present study. These specimens were demonstrated to be more efficient for use for PCR analysis than blood, allowing more patients without syphilis lesions to be studied.

We have identified five subtypes (subtypes 10a, 14a, 14c, 14f, and 14g) of *T. pallidum*, and with the exception of subtype 10a, all of these have been identified previously (4, 10, 12–14, 16). In the only *T. pallidum* subtyping study performed in Portugal (4), only three subtypes (subtypes 14a, 14d, and 14f) were found, while five subtypes were identified in the present study.

Taking into account the results of our study, it seems to us that the subtyping method used in the present study has good reproducibility and that in patients without lesions, the best specimen for use for the identification of the *T. pallidum* subtype is the ear lobe scraping, followed by plasma. The ear lobe has high number of capillaries and few sensory nerves, and scrapings are easily obtained. Therefore, collection of this specimen should be considered in future studies to confirm the consistency and the reproducibility of the results obtained with this specimen.

It should also be said that, to our knowledge, although other subtypes with the same number of *arp* repeats have been described previously (12, 14–16), subtype 10a was identified for the first time in this study. Therefore, further studies are needed to explain the presence of this subtype in Portugal, namely, to determine whether it has a possible relationship to the *Treponema pallidum* strains circulating in one or more of the African countries where Portuguese is spoken and where infected individuals have traveled.

REFERENCES

1. **Buchacz, K., J. D. Klauser, P. R. Kerndt, R. L. Shouse, I. Onorato, P. D. McElroy, J. Schwendemann, P. B. Tambe, M. Allen, F. Coye, C. Kent, M. N. Park, K. Hawkins, E. Samoff, and J. T. Brooks.** 2008. HIV incidence among men diagnosed with early syphilis in Atlanta, San Francisco, and Los Angeles, 2004 to 2005. J. Acquir. Immune Defic. Syndr. **47:**234–240.
2. **Direcção de Serviços de Informação e Análise, Divisão de Epidemiologia.** 2007. Doenças de declaração obrigatória 2002–2006. Regiões e sub-regiões de saúde no continente, regiões autónomas, p. 26. Direcção de Serviços de Informação e Análise, Divisão de Epidemiologia, Direcção-Geral da Saúde, Lisbon, Portugal. http://www.dgs.pt/, Estatisticas link.
3. **Fleming, D. T., and J. N. Wasserheit.** 1999. From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. Sex. Transm. Infect. **75:**3–17.
4. **Florindo, C., V. Reigado, J. P. Gomes, J. Azevedo, I. Santo, and M. J. Borrego.** 2008. Molecular typing of *Treponema pallidum* strains from Lisbon, Portugal. J. Clin. Microbiol. **46:**3802–3803.
5. Reference deleted.
6. **Hanson, J., S. Posner, S. Hassig, J. Rice, and T. A. Farley.** 2005. Assessment of sexually transmitted diseases as risk factors for HIV seroconversion in a New Orleans sexually transmitted disease clinic, 1990–1998. Ann. Epidemiol. **15:**13–20.
7. **Huhn, G. D., A. F. McIntyre, J. M. Broad, S. W. Holmes, A. Studzinski, C. Rabins, and M. S. Dworkin.** 2008. Factors associated with newly diagnosed human immunodeficiency virus among persons with concomitant sexually transmitted diseases. Sex. Transm. Dis. **35:**731–737.
8. **Liu, H., B. Rodes, C. Y. Chen, and B. Steiner.** 2001. New tests for syphilis: rational design of a PCR method for detection of *Treponema pallidum* in clinical specimens using unique regions of the DNA polymerase I gene. J. Clin. Microbiol. **39:**1941–1946.
9. **Martin, I. M., E. Foreman, V. Hall, A. Nesbitt, G. Forster, and C. A. Ison.** 2007. Non-cultural detection and molecular genotyping of Neisseria gonorrhoeae from a piece of clothing. J. Med. Microbiol. **56:**487–490.
10. **Molepo, J., A. Pillay, B. Weber, S. A. Morse, and A. A. Hoosen.** 2007. Molecular typing of *Treponema pallidum* strains from patients with neurosyphilis in Pretoria, South Africa. Sex. Transm. Infect. **83:**189–192.
11. **Morre, S. A., R. Moes, I. Van Valkengoed, J. P. Boeke, J. T. van Eijk, C. J. Meijer, and A. J. Van den Brule.** 1998. Genotyping of *Chlamydia trachomatis* in urine specimens will facilitate large epidemiological studies. J. Clin. Microbiol. **36:**3077–3078.
12. **Pillay, A., H. Liu, S. Ebrahim, C. Y. Chen, W. Lai, G. Fehler, R. C. Ballard, B. Steiner, A. W. Sturm, and S. A. Morse.** 2002. Molecular typing of *Treponema pallidum* in South Africa: cross-sectional studies. J. Clin. Microbiol. **40:**256–258.
13. **Pillay, A., H. Liu, C. Y. Chen, B. Holloway, A. W. Sturm, B. Steiner, and S. A. Morse.** 1998. Molecular subtyping of *Treponema pallidum* subspecies *pallidum*. Sex. Transm. Dis. **8:**408–414.
14. **Pope, V., K. Fox, H. Liu, A. A. Marfin, P. Leone, A. C. Sena, J. Chapin, M. B. Fears, and L. Markowitz.** 2005. Molecular subtyping of *Treponema pallidum* from North and South Carolina. J. Clin. Microbiol. **43:**3743–3746.
15. **Porras, C., M. Safaeian, P. González, A. Hildesheim, S. Silva, M. Schiffman, A. C. Rodríguez, S. Wacholder, E. Freer, K. Quint, C. Bratti, A. Espinoza, B. Cortes, R. Herrero, and Costa Rica HPV Vaccine Trial (CVT) Group.** 2008. Epidemiology of genital Chlamydia trachomatis infection among young women in Costa Rica. Sex. Transm. Dis. **5:**461–468.
16. **Sutton, M. Y., H. Liu, B. Steiner, A. Pillay, T. Mickey, L. Finelli, S. Morse, L. E. Markowitz, and M. E. St. Louis.** 2001. Molecular subtyping of *Treponema pallidum* in an Arizona county with increasing syphilis morbidity: use of specimens from ulcers and blood. J. Infect. Dis. **183:**1601–1606.