Detection of *Treponema pallidum* in Early Syphilis by DNA Amplification

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By using experimentally infected rabbits as a model for early syphilis, the applicability of in vitro DNA amplification was explored for detection of *Treponema pallidum*. It was determined that whole blood in heparin or EDTA (but not serum), lesion exudate, and punch biopsy as well as swabs of lesions are useful specimens for examination by the polymerase chain reaction. Swabs do not require special diluents, and the specimens, whether kept at room temperature or frozen, are well suited for use in the polymerase chain reaction.

Serologic tests for syphilis, which are very useful for confirmation of infection and follow-up of treatment, are insensitive during the early stage of primary syphilis or have unsatisfactory specificity in patients with congenital and neurosyphilis (6, 17). Although the detection of *Treponema pallidum* subsp. *pallidum* in patients with primary syphilis is the most reliable parameter of infection, available techniques are either insensitive (dark-field microscopy [17]) or impractical (rabbit infectivity test [RIT] [10, 18]). At present, DNA amplification by the polymerase chain reaction (PCR), the most sensitive and specific in vitro technique, has found growing application in the laboratory diagnosis of infectious diseases (4). In the case of syphilis, it has been applied for examination of cerebrospinal fluid (2, 5, 7, 15), amniotic fluid (2, 5), and neonatal blood serum (5), as well as for differentiation between *T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *pertenue* (14) and characterization of new treponemal isolates (13).

Using experimentally infected rabbits as a model for the early stage of syphilitic infection, we explored the applicability of the PCR in the detection of *T. pallidum* in blood and lesions. The purpose of these studies was to explore the feasibility of performing PCR with specimens collected and stored even under relatively adverse conditions in clinics or private offices located in areas remote from health-related laboratories where the technique may be available.

Male adult (New York State-Flemish Giant) rabbits (20) were infected intratesticularly (i.t.) with 3×10^7 organisms per testis or intradermally into the clipped skin of the back with 10^7 organisms per site (eight sites per animal). Primary dermal lesions were the sources of cotton swab (Cheston, Dayville, Conn.) smears, exudates, and punch biopsy specimens. Swabs from the abraded surfaces of early (10- and 18-day) lesions or taken after punch biopsies (18- and 30-day lesions) were kept in sterile screw-cap tubes (4 by 1 cm) without diluent (dry swabs) or were immediately immersed in 1 ml of either sterile phosphate-buffered saline (PBS) or TE buffer (containing 10 mM Tris and 1 mM EDTA [pH 8.0]). Punch biopsy specimens, obtained by using Acu-Punch (diameter, 3 mm; Acuderm, Fort Lauderdale, Fla.), or exudate fluids (10 to 20 μ l) were immediately mixed with 100 μ l of TE buffer. The specimens were kept at room

temperature ($22 \pm 2^\circ\text{C}$). Approximately 20 swabs were stored under more adverse conditions; namely, they were kept for 2 to 3 days at 37°C and then at -20°C prior to testing. Up to 8 swabs were taken from a single lesion, and 50 to 60 swabs were prepared from the lesions of a single animal and were tested at various times (0, 60, and 90 days) after collection. Peripheral blood from i.t.-infected animals was drawn into sterile tubes containing heparin (20 U/ml of blood) or EDTA (14 mg/5 ml of blood). Blood specimens were divided into aliquots with volumes of 1 ml and were immediately snap-frozen and kept at -30°C until they were tested. To compare the suitability of using whole blood versus serum, 18 blood samples were collected with or without heparin. The latter specimens were allowed to clot for 1 h at room temperature and for 4 h at 4°C before centrifugation and separation of serum. Whole blood and serum were then frozen until they were tested. All procedures, except taking of peripheral blood, were done with animals under anesthesia (Ketaset). Animal procedures were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center for Laboratories and Research.

Chromosomal DNA from Percoll-purified *T. pallidum* was isolated as described previously (15). Dilutions of *T. pallidum* DNA in TE buffer at concentrations of 250, 25, and 2.5 fg/5 μ l were used as controls of PCR sensitivity. DNA from *Haemophilus ducreyi* was kindly provided by Linda Parsons, DNA from herpes simplex virus was provided by Cinnia Huang, DNA from cytomegalovirus was provided by Greg Pari, and DNA from human immunodeficiency virus was provided by James Conroy; all are scientists at the Wadsworth Center for Laboratories and Research.

A standard suspension of 10^7 *T. pallidum* per ml was established by enumeration under a dark field (12) of six slides (5 μ l of treponemal suspension under a coverslip [22 by 22 mm]; 50 fields per slide; magnification, $\times 1,000$), and organisms were counted independently by three investigators. Aliquots containing 10^2 , 10^3 , and 10^4 organisms per ml in TE buffer and tRNA (Bethesda Research Laboratories, Gaithersburg, Md.), to be used as controls for DNA extraction, were kept at -30°C .

Samples for PCR were prepared as follows. For cell lysis, 50 μ l of whole blood, serum, or lesion exudate was mixed with 50 μ l of $2\times$ lysis buffer (LB; $1\times$ LB contains 50 mM Tris-HCl [pH 8.5], 50 mM NaCl, 4 mM MgCl_2 , 0.01% sodium dodecyl sulfate, and proteinase K [100 $\mu\text{g/ml}$]).

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TABLE 1. Specimens from experimentally infected rabbits used for evaluation of syphilis by PCR

<i>T. pallidum</i> source	Specimen	Preservation conditions ^a	PCR results at the following time (days) after collection ^b :		
			0	60	90
Lesions at early development (10 days)	Swabs ^c	TE buffer ^d	4/4	ND	7/8
Lesions at peak of ulceration (18 days)	Swabs	No diluent	5/5	3/3	ND
	Swabs	PBS	9/9	7/7	6/8
	Swabs	TE buffer	5/5	3/3	ND
	Exudate	TE buffer	4/4	4/4	6/8
	Punch biopsy (diameter, 3 mm)	TE buffer	4/4	4/4	7/7
Healing lesions (30 days)	Swabs	PBS	5/8	3/8	1/10
	Punch biopsy (diameter, 3 mm)	TE buffer	6/8	ND	10/12

^a All specimens were kept at room temperature.^b Values are number of positive samples/total of samples tested. For data at time zero, PCR was done on the day of collection. ND, not done.^c Swabs were taken from the surfaces of abraded lesions. In other animals, the swabs were taken from inside the lesions after punch biopsy.^d Tris-EDTA buffer concentrated two times.

Tissue biopsy specimens were firmly pressed with a pipette tip into 100 μ l of 2 $\times$ LB and vigorously vortexed. To the swabs immersed in 1-ml diluents (PBS or TE buffer), 10 μ l of 100 mM EDTA (pH 8.0), 10 μ l of 10% sodium dodecyl sulfate, and 10 μ l of proteinase K (10 mg/ml) were added and vortexed vigorously. To the dry swabs, 1 ml of TE buffer was added prior to examination, vortexed, and processed as described above for the swabs kept in diluents. From each specimen, 50 μ l of fluid was mixed with 50 μ l of 2 $\times$ LB. To the swab specimens and *T. pallidum* suspensions containing a low concentration of eukaryotic DNA, 100 μ g of tRNA per ml was added as a carrier. Treated specimens were incubated for 1 to 2 h at 55°C or overnight at 37°C, followed by 15-min heating at 100°C to inactivate proteinase K, and were kept on ice until further processing or were frozen until use. After centrifugation for 2 min at 500 $\times$ g to remove all cell debris, DNA extraction was carried out by the method of Boom et al. (1) by using 50 μ l of the lysate adsorbed to diatoms in the presence of guanidine thiocyanate as described previously (15). In each series of extractions, positive (*T. pallidum* aliquots) and negative controls were included.

For in vitro DNA amplification, the protocol used was nearly identical to that described previously (15) and was based on the *bmp* gene of *T. pallidum* (3), with one modification; namely, tetramethylammonium chloride (0.05 mM) was added to the PCR mixture as an enhancer (9). Primers Tp3, Tp4, Tp7, and Tp8 were synthesized (15) or were produced at the Wadsworth Center for Laboratories and Research in a DNA synthesizer (381A; Applied Biosystems, Foster City, Calif.). Briefly, 30 cycles of PCR 1 with primers Tp7 and Tp8 produced 617-bp fragments. After dilution to 1:10 (distilled water), PCR 2 (nested PCR) was done with Tp3 and Tp4 to produce a 500-bp fragment. For each run, positive (chromosomal DNA) and negative (mixture of reagents) controls were included. PCR products were analyzed in 2% agarose (Seakem, Rockland, Maine) containing ethidium bromide (Sigma, St. Louis, Mo.).

To avoid contamination of reagents and samples with stray DNA, four laboratories located on three different floors of the building were used for (i) preparation of reagents, (ii) preparation of the PCR mixture and DNA extraction (in a biologic safety hood), (iii) amplification and dilution of samples for nested PCR, and (iv) analysis of the PCR

products. Labware, reagents, and laboratory coats were separate for each laboratory.

The primers for PCR 1 and PCR 2 were specific for *T. pallidum* DNA. No amplification with DNA of possible skin contaminant microorganisms was found when the method was examined previously (15); and no amplification associated with microorganisms causing sexually transmitted diseases, other than syphilis, such as *H. ducreyi*, cytomegalovirus, herpes simplex virus, and human immunodeficiency virus used in the present experiments, was found.

When *T. pallidum* of various concentrations was used, DNA equivalent to 2 $\times$ 10² organisms per ml, or 1 organism per 5 μ l, was consistently detected. However, when fresh normal blood samples were seeded with various concentrations of *T. pallidum* and examined immediately, the threshold of detection increased (DNA equivalent to $\geq$ 10³ organisms per ml). Although the extraction of DNA used in our procedure was assumed to minimize the inhibitory effect of leukocytes (2, 15), it apparently did not totally prevent it.

Swabs taken during the development of primary lesions at 10 and 18 days postinfection, with or without diluents and kept at room temperature for 60 days in tightly closed tubes, were 100% positive by PCR (Table 1). A large percentage of swabs taken from lesions 10 or 18 days postinfection and immersed in TE buffer (87%) or PBS (75%) were still positive after storage for 90 days at room temperature. At 30 days after infection, the number of treponemes in the healing lesions had decreased so far that in only five of eight lesions (63%) was *T. pallidum* detectable by PCR; after storage for 90 days, only 10% of the samples were still positive.

Sixteen exudate samples were examined by PCR on the same day or were kept at room temperature until they were tested. All four samples (100%) examined on the same day or on day 60 after collection and six of eight (75%) samples examined on day 90 after collection were positive by PCR (Table 1).

All 15 punch biopsy specimens of early lesions (obtained 18 days after infection), regardless of the time of examination, were PCR positive. Punch biopsy specimens from healing lesions (obtained on day 30 after infection) proved to be a relatively better specimens than swabs taken from the same lesions, whether they were examined on the same day as sampling (75% were PCR positive) or 90 days after collection (83% were PCR positive) (Table 1). Interestingly,

FIG. 1. Amplification by PCR 1 and PCR 2 of *T. pallidum* DNA from various specimens taken from infected rabbits. The amplified products were from biopsy specimens of tissues of healing skin lesions (30 days) (lanes 1 to 4), early lesions (10 to 18 days) (lanes 5 to 8), swabs of abraded early lesions (lanes 10 to 16), whole blood of i.t.-infected rabbits (lanes 18 to 20). Lanes 9 and 17 are reagent (negative) controls.

eight skin biopsy specimens taken 4 months postinfection (at the eight sites of infection) and examined by PCR on the same day were all negative for *T. pallidum* DNA (data not shown).

The suitability of whole blood as a potential specimen to be tested by PCR was also examined. Blood from 10 i.t.-infected rabbits was drawn into heparin or EDTA tubes or without anticoagulants (for serum) every 3 days for a period of 12 days. Regardless of the anticoagulant used, *T. pallidum* DNA was detected in 3 of 10 (30%) of the animals on day 4 postinfection and in 9 of 10 (90%) of the animals on day 8 postinfection. The animal whose blood was negative by PCR did not develop orchitis. Of 18 serum samples from animals whose whole blood was PCR positive, only 1 (6%) gave a positive PCR result. Some of the DNA-amplified products of tissue biopsy specimens, swabs, and blood are shown in Fig. 1.

In the presence of a high concentration of the chaotropic agent guanidine thiocyanate, the DNA readily binds to diatomaceous earth, facilitating its extraction and purification from potential inhibitory substances. The yield of DNA by use of diatoms is higher (1) than that by use of the combination of phenol extraction and alcohol precipitation (2, 5, 7). This and the performance of PCR with nested primers provided a very specific and sensitive method for the detection of *T. pallidum* DNA. Using nested PCR, we were able to detect 1 to 2.5 fg of DNA from Percoll-purified *T. pallidum*, or 1 organism in 5 μ l, which is equivalent to 200 organisms per ml. Extraction of larger volumes of samples, e.g., 250 μ l (15), yields an increase in the sensitivity. The sensitivity in our experiments was similar to that reported by Burstein et al. (2) and Hay et al. (7), who reported that 1 to 10 organisms per 50 μ l of sample were needed for a positive PCR.

In these studies, confirmation of the specificity of the PCR assay by hybridization with *T. pallidum*-specific oligonucleotide was considered unnecessary since the animals were infected with a known microorganism. In addition, in the nested PCR, amplification with the second set of primers does not occur if the product of the first amplification is not specific for *T. pallidum*.

Conditions of storage for specimens obtained during early experimental syphilis were less stringent than those suggested for material obtained from cerebrospinal fluid or from patients with congenital syphilis (5). We assumed that the large number of treponemes present in primary lesions or during spirochetemia was more than sufficient to compensate for losses inherent under less than appropriate storage conditions. It is known that lesions begin to appear at the site of infection when the number of organisms is on the order of 10^7 treponemes (19); when the lesion is fully developed, a

much larger number of organisms must be available. On the other hand, samples from healing lesions, where clearance of treponemes has already taken place, were less suitable for examination by means of swabs. Biopsy specimens provided better results than swabs did, particularly when examination could not be done on the same day of specimen collection. However, at the same time that the primary lesion subsides, treponemal and phospholipid antibodies are present in 75 to 90% of the patients and the serodiagnosis is a helpful adjunct to support clinical diagnosis. Under such circumstances, no PCR would be required. However, in some cases of human immunodeficiency virus-*T. pallidum* coinfections, the serodiagnosis may be noncontributory and PCR with biopsy tissue could be helpful in diagnosing a dual infection.

Some losses of *T. pallidum* during storage at room temperature must be expected; this can be improved by storage of the samples at lower temperatures, including freezing, as was done with the blood specimens in this study. In this study, no obvious differences were noticed when swabs were placed in dry tubes or in PBS or TE buffer. We assume that specimens from the anal region or oral cavity, with which diagnosis by microscopy is difficult because of contamination with nonpathogenic spirochetes (17), can also be examined by PCR. Moreover, the specific in vitro DNA amplification method takes greater importance in view of a recent report (16) indicating that pathogen-related spirochetes, which cause acute necrotizing ulcerative gingivitis, reacted with monoclonal antibodies that were once considered to be pathogen specific (*T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *pertenue*) (11).

Blood specimens obtained with heparin, which is reported to have an inhibitory effect on gene amplification (8), did not prevent us from obtaining results as good as those obtained with blood taken into EDTA tubes, probably owing to the DNA extraction method used.

Unlike whole blood, serum specimens were unsuitable for examination by PCR. The only PCR-positive serum specimen was partially hemolyzed. These results disagree with those recently published by Grimpel et al. (5), who reported that 9 of 21 (43%) serum specimens from neonates with congenital syphilis were positive by PCR and 6 of 6 serum specimens were positive by RIT. Although we did not examine our specimens by RIT, our PCR results are consistent with those of Turner and Hollander (18), who reported positive RIT results in 5 of 13 (38%) whole blood samples and none of the serum samples examined. Apparently, in our experiments, treponemes were efficiently trapped during the clotting process. Older literature (quoted in reference 18) also stresses the unsuitability of sera for examination by RIT.

An animal model with susceptibility to infection similar to

that of humans proved to be of great help in assessing the usefulness of the PCR for diagnosis during early syphilis. The model provided information obtained under controlled experimental conditions, allowing for the use of multiple and various collections of specimens, and was helpful in tracing the pathogen during the process of hematogenous dissemination and clearance from the lesions during the healing process.

The combination of both in vitro DNA amplification and animal models may provide additional help in the progress of laboratory diagnosis of congenital syphilis and neurosyphilis and in assessing the efficacy of antibiotic treatment.

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