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Effects of Various Handling and Storage Conditions on Stability of *Treponema pallidum* DNA in Cerebrospinal Fluid

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Treponema pallidum **DNA from even small numbers of organisms was detectable in cerebrospinal fluid (CSF) stored at room temperature or at 4°C for several hours and in CSF subjected to three freeze-thaw cycles. These results suggest that negative PCR results for *T. pallidum* from patients diagnosed with *T. pallidum* invasion of the central nervous system are probably not due to the loss of target DNA prior to testing.**

Treponema pallidum subsp. *pallidum* can invade the central nervous system (CNS) at various stages of the disease (2, 11). CNS invasion by *T. pallidum* is determined by a positive syphilis serologic test, abnormal cerebrospinal fluid (CSF) cell count and protein level, and/or a positive CSF Venereal Disease Research Laboratory (VDRL) test. Despite the high specificity of the CSF VDRL test, its sensitivity ranges from 22 to 69% in patients with symptomatic neurosyphilis (5). There are conflicting reports pertaining to the utility of PCR to help diagnose CNS invasion and other manifestations of infection with *T. pallidum* (1, 3, 4, 6, 7, 9, 10).

The objective of this study was to determine how exposure of CSF to various environmental conditions affects the ability of a highly sensitive PCR assay to detect *T. pallidum* DNA in CSF samples spiked with spirochetes. We observed that *T. pallidum* DNA, from even small numbers of organisms, was detectable in CSF stored at room temperature or 4°C for several hours and in CSF subjected to multiple cycles of freeze-thawing.

T. pallidum subsp. *pallidum* Nichols, frozen on dry ice at a concentration of 5×10^7 spirochetes per ml (quantitated microscopically in the provider's laboratory prior to shipment), was generously provided by Sheila A. Lukehart (University of Washington, Seattle). Upon arrival in our laboratory, one tube of spirochetes was thawed to 4°C, mixed well, and diluted to 500,000 spirochetes per ml of phosphate-buffered saline (PBS). Serial dilutions in PBS that corresponded to 5,000, 500, 50, 5, and 0.5 spirochetes per 100 μl of diluent were made from the initial dilution. DNA was extracted from the specimens with the IsoQuick nucleic acid extraction kit (MicroProbe, Bothell, Wash.). The procedure for sample lysis and rapid DNA extraction recommended by the manufacturer was followed with the following exceptions: following the addition of 0.1 volume of sodium acetate to the aqueous phase of the extract, 2.0 μl of glycogen (Boehringer Mannheim Corp., Indianapolis, Ind.) was added, followed by a volume of ice-cold isopropanol. The sample was then stored at −20°C for 30 min and centrifuged at 12,000 × *g* for 15 min at room temperature. The entire contents of each extraction product were used in each amplification reaction mixture. Amplification by PCR of a 658-bp portion of the *T. pallidum* 47-kDa membrane immunogen gene (nucleotides 648 to 1305) was based upon the methods of Burstain et al. (1), with the following modifications: for each amplification reaction mixture, dUTP was used at a final concentration of 400 μM in place of dTTP, glycerol was used at a final concentration of 10%, and Isopsoralen IP-10 (HRI Associates, Inc., Concord, Calif.) was used at a final concentration of 25 μg/ml. Following amplification and postamplification inactivation, 20 μl of each reaction mixture was analyzed by electrophoresis using a 3% agarose gel (2% SeaKem LE agarose and 1% NuSieve GTG agarose; FMC BioProducts, Rockland, Maine) containing 0.5× Tris-borate-EDTA buffer and 0.16 mg of ethidium bromide per ml. The gels were photographed under UV illumination, and the DNA was transferred to a nylon membrane (Hybond-N; Amersham Life Science, Arlington Heights, Ill.) by Southern blotting. The membranes were prehybridized and then hybridized with a DNA probe prepared as described by Burstain et al. (1), using the procedure described for the Enhanced Chemiluminescence Direct Nucleic Acid Labeling and Detection System (Amersham Life Science). A rotisserie hybridization oven (Hybaid; National Labnet Co., Woodridge, N.J.) was used for all prehybridization and hybridization steps. The blots were developed by the procedure for the Amersham Enhanced Chemiluminescence Direct Nucleic Acid Labeling and Detection System. *T. pallidum* DNA was detected in all dilutions of spirochetes except the dilution corresponding to 0.5 spirochete per 100 μl of PBS (Fig. 1A). To confirm and extend these findings, spirochetes were serially diluted in pooled VDRL test-negative CSF at concentrations of 1,000, 100, 50, 10, 5, 1, and 0.1 spirochetes per 100 μl. The use of spent patient CSF for these experiments was approved by the Human Investigation Committee at Wayne State University. *T. pallidum* DNA was detected in all spiked CSF dilutions except the 1 and 0.1 spirochete dilutions (Fig. 1B). The results from these two experiments confirm the concentration of spirochetes determined microscopically by an outside laboratory and demonstrate that the assay is as sensitive in our laboratory as previously reported by Burstain et al. (1). The spirochetes were stored in equal volumes of 50:50 rabbit serum-saline solution and sterile glycerol at −70°C. Prior to the performance of any subsequent experiments with the spirochetes, the concentration of the spirochetes was always estimated by using the limiting dilution PCR procedure described above to ensure the use of consistent concentrations of organisms. In addition, phase-contrast microscopy was also done to confirm the presence of the spirochetes in the stored aliquots.

To determine how various environmental conditions affected the ability of PCR to detect *T. pallidum* DNA in CSF, 100-μl aliquots of CSF were spiked with known quantities of

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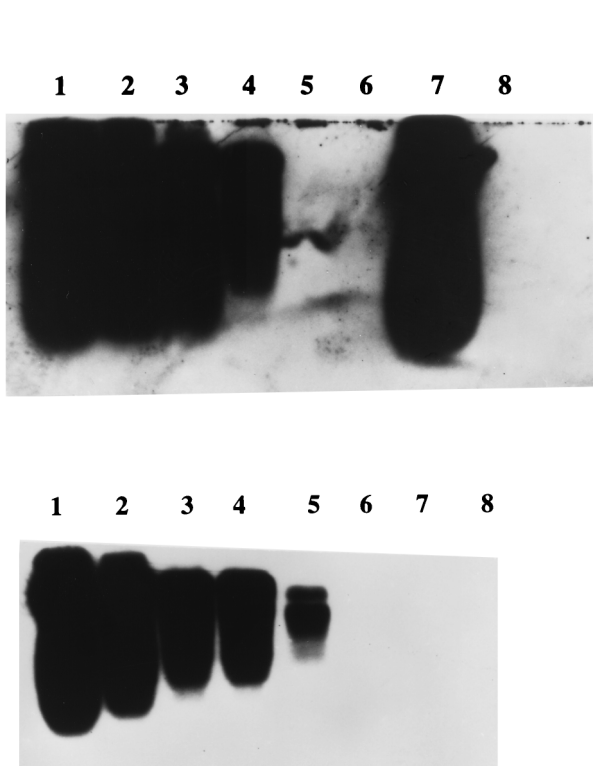

FIG. 1. Southern blot analysis showing the sensitivities of the *T. pallidum* PCR assay. (A) Lanes: 1 to 5, 5,000, 500, 50, 5, and 0.5 spirochetes, respectively, per amplification reaction mixture diluted in PBS prior to extraction of nucleic acids; 6, PBS extraction blank (no target); 7, plasmid positive control (American Type Culture Collection, Rockville, Md.); 8, amplification reagent blank (no target). (B) Lanes: 1 to 7, 1,000, 100, 50, 10, 5, 1, and 0.1 spirochetes, respectively, per amplification reaction mixture diluted in CSF prior to extraction of nucleic acids; 8, CSF extraction blank (no target).

spirochetes and then subjected to various storage and handling conditions. In three separate experiments, CSF was spiked with approximately 500, 50, 25, 10, and 5 organisms per aliquot and stored at room temperature for various time periods up to 96 h. After DNA extraction and PCR amplification, *T. pallidum* DNA was detected in all aliquots stored at room temperature (Table 1). To determine how storage at 4°C affects PCR detection of *T. pallidum* DNA in CSF, approximately 500, 50, 25, 10, and 5 spirochetes per 100 μl of CSF were stored in a refrigerator for various time periods up to 96 h. In two separate experiments, *T. pallidum* DNA was detected by PCR in all aliquots stored at 4°C (Table 1). Freezing-thawing is a common occurrence in stored samples when multiple procedures are performed on a specimen over a period of time. CSF was spiked with approximately 500, 50, 25, 10, and 5 spirochetes per 100-μl aliquot, stored at −20°C for 1 to 2 h, and then thawed to room temperature. This procedure was repeated for three freeze-thaw cycles. An aliquot was taken after each freeze-thaw cycle, and DNA was extracted. In two separate experiments, *T. pallidum* DNA was detected in aliquots at all concentrations tested except the 25-spirochete/μl aliquot in the third cycle of one experiment (Table 2). This discrepancy may have been caused by a sampling or dilution error during the preparation of the aliquot.

TABLE 1. Detection of *T. pallidum* in CSF after storage at room temperature and 4°C

Temp	No. of organisms[a]	Detection[b] after storage time (h) of:					
		0	4	8	24	48	96
Room	500	+++	[c]++	++	++	++	++
	50	+++	++	++	++	++	++
	25	++	++	++	++	+	+
	10	++	++	++	++	+	+
	5	+	ND[c]	ND	ND	+	+
4°C	500	+	+	+	+	+	+
	50	++	+	+	++	++	++
	5	++	+	+	++	++	++
	10	+	+	+	+	+	+

[a] Estimated number of spirochetes per amplification reaction mixture diluted in 100 μl of CSF prior to initiation of the experiment.
[b] *T. pallidum* DNA detected by PCR, with each + representing an independent experiment; i.e., ++ = *T. pallidum* DNA detected in two independent experiments.
[c] ND, not done.

TABLE 2. Detection of *T. pallidum* in CSF after freezing and thawing

No. of organisms	Detection[a] after freeze-thaw cycle(s) numbering:			
	0	1	2	3
500	++	++	++	++
50	++	++	++	++
25	+	+	+	—[b]
10	+	+	+	+
5	+	+	+	+

[a] See Table 1, footnote *b*, for explanation of symbols.
[b] —, no *T. pallidum* DNA detected by PCR.

Variable results have been reported for PCR detection of *T. pallidum* in the CSF of patients suspected of having CNS invasion by *T. pallidum* (3, 6, 7, 9, 10). The reasons for the differences in the results reported in the literature are not clear. Concerns regarding the impact that specimen handling may have on the outcome of PCR testing for *T. pallidum* in patient specimens have been mentioned by several investigators (4, 8, 10, 12). Our study demonstrates that *T. pallidum* DNA can be detected in CSF by PCR after the CSF has been subjected to various environmental and handling conditions that could be encountered in a clinical setting. These results suggest that the negative PCR results reported for clinical specimens from patients diagnosed with CNS invasion by *T. pallidum* could be due to the absence of organisms in the CSF, to the presence of only a minimal number of organisms, or to the presence of inhibitors to PCR amplification in the patient specimen or specimen extract.

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