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Use of Polymerase Chain Reaction and Rabbit Infectivity Testing To Detect *Treponema pallidum* in Amniotic Fluid, Fetal and Neonatal Sera, and Cerebrospinal Fluid

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The diagnosis of congenital syphilis continues to pose a difficult clinical challenge. Because the serodiagnosis of congenital syphilis has significant limitations, the direct detection of *Treponema pallidum* in suspect neonatal tissues or body fluids represents a desirable alternate diagnostic strategy. We developed and applied the polymerase chain reaction (PCR) for the detection of *T. pallidum* in clinical materials relevant to the diagnosis of congenital syphilis but which typically contain factors inhibitory for the PCR. Four methods of specimen processing were examined to circumvent PCR inhibition; clinical materials included amniotic fluids, neonatal sera, and neonatal cerebrospinal fluids. The PCR was 100% specific for *T. pallidum* compared with the sensitive rabbit infectivity test (RIT) for all clinical materials tested. For amniotic fluids, the PCR was 100% sensitive when correlated with the RIT but had a lesser sensitivity when applied to sera or cerebrospinal fluids, which typically contain few treponemes. The combined sensitivity of the PCR for all clinical samples was 78%. Positive PCR results also were obtained among some clinical specimens for which RIT was not performed; these results correlated well with either stigmata or risk factors for congenital syphilis. The combined results suggest that the PCR can be a useful adjunct to the diagnosis and clinical management of congenital syphilis and that it will provide a valuable tool for investigations of the pathogenesis of the disorder.

There has been an alarming resurgence of syphilis in the United States (8, 9). As a predictable consequence of the increased incidences of primary and secondary syphilis in women of childbearing age (8, 9, 11, 12), rates of congenital syphilis in the United States also have risen sharply (9, 11). The current epidemic of congenital syphilis has heightened the awareness of clinicians and researchers of the diagnostic and therapeutic dilemmas concerning this disease (43).

The diagnosis of congenital syphilis remains a significant clinical challenge (24, 27, 37). *Treponema pallidum* subsp. *pallidum* cannot be cultivated in vitro, and identification of the pathogen in suspect neonatal tissues or body fluids can rarely be accomplished at birth because of a paucity of organisms in clinical specimens and/or the limited sensitivity of routinely available methods for detection of the organism (e.g., dark-field microscopy) (25). The most sensitive and reliable method for detecting *T. pallidum* in clinical specimens has been the rabbit infectivity test (RIT) (34, 35); RIT provides definitive evidence of viable *T. pallidum*, and it is considered to be the current standard method for *T. pallidum* detection (34, 35). However, use of the RIT as a routine diagnostic procedure for congenital or adult syphilis is impractical. Serological testing has been used as an adjunct to the diagnosis of congenital syphilis; the central problem with the serodiagnosis of congenital syphilis is distinguishing the asymptomatically infected infant from the uninfected infant born to a mother with reactive syphilis serological tests. This difficulty stems largely from the inability to distinguish the humoral immune response of the mother, whose immunoglobulin G (IgG) antibodies pass transplacentally to the fetus, from the specific antibody response of the infant (27).

Maternal IgM does not cross the human placenta, and thus the assessment of total fetal IgM levels has been another strategy used in the diagnosis of congenital syphilis (1, 16, 28, 38). However, this approach is nonspecific (2, 3, 26, 31, 39). Methods for detecting specific fetal IgM antibodies directed against *T. pallidum*, such as the fluorescent treponemal antibody-absorption IgM test, also have not been reliable because of a lack of sensitivity and specificity (28). Detection of fetal IgM directed specifically against selected immunogens of *T. pallidum* by Western blot (immunoblot) analysis has shown promise (14, 33, 42), but its level of sensitivity is unknown.

Recently, we developed a sensitive and specific assay for *T. pallidum* by using the polymerase chain reaction (PCR) (7); PCR proved to be exquisitely sensitive for detecting purified *T. pallidum* or *T. pallidum* DNA. However, inhibition of the enzymatic amplification of *T. pallidum* DNA was found to be an unexpected problem when the PCR method was applied to clinical specimens (7). This prompted us to undertake a study with two principal objectives: (i) to develop methodologies for processing specimens for PCR in order to overcome PCR inhibition and (ii) to compare retrospectively the results of PCR with RIT data obtained for specimens collected as part of our ongoing congenital syphilis program. The specimens available for these analyses were amniotic fluids collected from pregnant women with untreated syphilis and sera and cerebrospinal fluids (CSF)

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from neonates with probable or suspected congenital syphilis. We explored relatively simple methodologies for clinical specimen preparation that largely overcame PCR inhibition. The PCR promises to be a valuable diagnostic and clinical research tool for investigations of congenital syphilis.

MATERIALS AND METHODS

Patients. Informed consent was obtained from the patients or their parents or guardians, and the human study protocols were approved by the University of Texas Southwestern Medical Center Institutional Review Board according to U.S. Department of Health and Human Services guidelines. All pregnant women enrolled in this study were diagnosed with untreated early syphilis either during pregnancy or at delivery on the basis of conventional diagnostic criteria (29). All neonates underwent physical examinations for signs of congenital syphilis, CSF analyses (Venereal Disease Research Laboratory test [VDRL], cell count, and protein concentration determination), and long-bone radiographic surveys for osteochondritis or periostitis; infants had a diagnosis "compatible" with congenital syphilis according to the Centers for Disease Control classification (10). Control patients consisted of pregnant women and healthy full-term newborns with no history of syphilis and with nonreactive serological VDRL tests and microhemagglutination assays for *T. pallidum* antibodies. Amniotic fluids, sera, and CSF from these patients were used as negative controls in selected experiments throughout the studies.

Clinical samples. Amniotic fluids (5 to 10 ml) were obtained at various gestational ages during the pregnancy to establish fetal lung maturity by sonographically directed amniocentesis. Specimens were subjected to dark-field microscopic examination within 30 min after collection, and 2 ml of amniotic fluid was used for rabbit infectivity testing (see below). The remaining amniotic fluids were aliquoted in 1.5-ml microcentrifuge tubes and stored at −70°C until further use for PCR analysis.

CSF (50 to 200 μl) and blood (100 μl to 2 ml) were obtained from newborns (before antibiotic treatment) 1 to 3 days after delivery. Sera were collected by centrifugation (10 min at 500 × *g*) after clotting. Both CSF and sera were stored at −70°C from 1 to 7 days after collection until analyzed by PCR.

Dark-field microscopy. Amniotic fluids were examined by dark-field microscopy for the presumptive identification of *T. pallidum* by typical morphology and motility (17, 25, 45, 46). Ten microliters of each sample was placed on an acid-washed microscope slide with a coverslip and examined for 10 to 30 min at ×400 magnification and under oil immersion (×900). The detection of at least one typical motile organism by several observers was sufficient to establish positivity of the test results. Dark-field microscopy was not performed on sera or CSF.

RIT. RIT was carried out as described by Lukehart et al. (34). Briefly, 0.5 to 1.0 ml of each sample was injected intratesticularly into seronegative New Zealand White adult male rabbits. Rabbits were fed antibiotic-free food and water and were housed in an 18 to 20°C environment. The animals were examined for the development of orchitis and were bled for rapid plasma reagin tests and microhemagglutination assays for *T. pallidum* antibodies every 3 weeks for 3 months. When infection was suspected because of seroconversion or development of orchitis, a testicular aspiration was performed for dark-field examination. If the sample was negative by dark-field examination, the rabbit was sacrificed and the testes and popliteal lymph nodes were surgically removed and minced in 50% normal rabbit serum–50% isotonic saline solution for treponemal extraction. The extract was centrifuged for 7 min at 500 × *g*, and the supernatant was examined by dark-field microscopy. If the sample was again negative, 1.0 ml of the testicular extract was injected into each testis of a second seronegative rabbit according to the same procedure.

Positivity by RIT was established by identification of motile spirochetes by dark-field microscopy of a testicular aspirate (or at orchiectomy in the first or second rabbit). RIT results were negative if seroconversion or orchitis did not occur by 3 months after inoculation of the specimen.

DNA preparation for PCR. Four different methods of DNA preparation were investigated in order to determine the best method for circumventing PCR inhibition encountered in clinical specimens. (i) For the boiling method, 10 or 20 μl of serum or CSF was placed in a 1.5-ml microcentrifuge tube, subjected to boiling in a water bath for 10 min, and then cooled on ice and centrifuged for 10 s at 13,000 × *g* before further processing by PCR. In the case of sera, the whole coagulum obtained after boiling was placed into the PCR mixture and fragmented through the mineral oil by using the end of a micropipette tip. (ii) For low-spin separation, 100 μl of amniotic fluid, serum, or CSF was added to 900 μl of sterile phosphate-buffered saline (in 1.5-ml microcentrifuge tubes) and was centrifuged for 10 min at 1,000 × *g* (room temperature). The supernatant was placed into another microcentrifuge tube and centrifuged for 1 h at 20,000 × *g* (4°C). The second supernatant was discarded, the pellet was resuspended in 50 μl of sterile water and immediately boiled for 10 min and cooled on ice, and 40 μl of the suspension was used for PCR. This method is analogous to that described by Malloy et al. (36), who applied PCR for the detection of *Borrelia burgdorferi*. (iii) The alkaline lysis extraction method has been previously described (7); briefly, 100 μl of amniotic fluid, serum, or CSF was boiled for 1.5 min in 1 M NaCl, 1 N NaOH, and 0.1% sodium dodecyl sulfate (SDS). The volume was then neutralized with 400 μl of 0.5 M Tris-HCl (pH 8.0) and was extracted successively with 1 volume of phenol; the phenol was then reextracted with 100 μl of 0.15 M NaCl. The entire 600 μl was extracted with 1 volume of chloroform-isoamyl alcohol (24:1) (vol/vol) before precipitation with 1 volume of isopropyl alcohol (overnight at −20°C). The tubes were centrifuged for 15 min at 13,000 × *g* (4°C), decanted, and dried. The pellets were resuspended in 51 μl of sterile water. Of the 51 μl, 1, 10, and 40 μl each of the resulting DNA solution were subjected separately to PCR analysis. (iv) The spin extraction method was used for selected samples and consisted first of a low-spin separation (described above), followed by 10 min of boiling and one phenol-chloroform extraction (1:1, vol/vol) before precipitation in 2 volumes of cold absolute ethanol. The remainder of the procedure was identical to the alkaline lysis method. The entire DNA solution (50 μl) was subjected to PCR analysis. Each experiment was limited to eight samples, including a negative control which was used to assess spurious DNA contamination during the procedure.

PCR. Amplification of the 658-bp portion of the gene encoding the 47-kDa membrane lipoprotein immunogen has been previously described (7). Briefly, 100 μl of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 100 μg of gelatin per ml, 0.5 μg each of primers 47-1 and 47-2 (Table 1), 300 mM deoxyribonucleotides, 2.5 U of *Taq* polymerase (Perkin-Elmer/Cetus, Norwalk, Conn.), and various amounts of different DNA preparations was

TABLE 1. Sequences of oligonucleotide primers

Primer	Sequence	Coding strand	Location (nt)[a]
47-1	GACAATGCTCACTGAGGATAGT	+	648–669
47-2	ACGCACAGAACCGAATTCCTTG	−	1284–1305
47-3	TTGTGGTAGACACGGTGGGTAC	+	713–734
47-4	TGATCGCTGACAAGCTTAGGCT	−	1187–1208

[a] nt, nucleotides.

maintained for 1 min and 15 s at 94°C, 1 min at 60°C, and 1 min at 72°C for 40 cycles in a thermocycler (Perkin-Elmer/Cetus) without any final additional extension step and were then stored at 4°C until analyzed. The DNA preparations were added to the PCR tubes with either standard sterile 200-μl tips plugged with glass wool or 25-μl positive displacement tips (Rainin). To minimize the risk of contamination, all PCR reagents and the PCR tubes were prepared under a laminar flow hood in a DNA-free area remote from the principal laboratory. The DNA preparation and addition of the DNA to the PCR mixture were performed in another PCR-dedicated facility systematically cleaned with undiluted bleach and exposed to UV light for at least 12 h between each experiment. Each PCR experiment included two reagent control tubes; the negative control consisted of sterile water added through the mineral oil, and the positive control contained 10 μl of 0.1 μg/μl PCR-amplified plasmid pPH47.2 (23), added just before amplification (in a third room remote from the two areas previously mentioned).

Dot blot hybridization. One-tenth of the PCR product (10 μl) was chemically denatured for 10 min in 10 μl of 0.5 M NaOH–1.5 M NaCl, neutralized with 380 μl of 1.5 M NaCl–1 M Tris (pH 7.2), and blotted onto nitrocellulose by using a Minifold I apparatus (Schleicher & Schuell). The blots were baked for 2 h at 80°C under vacuum and hybridized overnight at 65°C with an internal 496-bp probe (7) generated by using primers 47-3 and 47-4 in a PCR reaction (Table 1). The probe was then labeled by random priming (Boehringer-Mannheim, Indianapolis, Ind.) (7). The next day, the blots were washed successively in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 15 min three times at room temperature and twice at 65°C. Autoradiography was performed at room temperature on Fuji X-ray film for 2 h to 2 days, depending on the specific activity of the probe.

RESULTS

Amniotic fluids. Because of increasing evidence that the identification of *T. pallidum* in amniotic fluid is an accurate predictor of congenital infection (17, 25, 45, 46), amniotic fluids from 11 serologically reactive pregnant women with untreated syphilis and 1 healthy control were examined for *T. pallidum* by various methods (Table 2). Nine of these 11 specimens were tested by RIT at the time of collection; 7 of the 9 specimens were RIT positive (Table 2, no. 1 to 7). Of those seven RIT-positive specimens, only five (71%) were positive by dark-field microscopy, but all seven were positive by PCR using one or more methods of specimen preparation (Table 2). The two RIT-negative (no. 8 and 9) amniotic fluids, obtained from patients with early primary syphilis, were negative by PCR, regardless of the PCR protocol used (Table 2). Positive PCR results also were obtained for two additional specimens (no. 10 and 11) not analyzed by RIT (Table 2); dark-field microscopy was performed on these two specimens and was positive only for patient 11, who delivered a symptomatic infant (as did patient 15, shown in Table 3). The infant of patient 10 was completely asymptomatic at delivery. The last specimen (no. 12), obtained from a seronegative healthy pregnant woman, was negative by PCR and RIT. Four additional amniotic fluids obtained from nonsyphilitic pregnant women also were negative by all PCR methods and by RIT (data not shown).

The low-spin separation method for PCR processing of amniotic fluids gave the best correlation (100%) with the RIT, although the alkaline lysis method missed only one case (no. 3). All four methods of sample processing revealed some positive PCR amplifications among specimens 10 and 11 (Table 2), but the low-spin separation and alkaline lysis PCR protocols each were positive for both. The spin extraction method of PCR was performed on four specimens for

TABLE 2. PCR detection of *T. pallidum* in amniotic fluids of women with untreated syphilis

Patient no.	Maternal stage[a]	Serum VDRL titer	Results with amniotic fluids[b]								
			DF	RIT	PCR	PCR method					
						B	S	SE	AL		
									1 μl	10 μl	40 μl
1	1°	1:64	+	+	+	+	+	ND	+	+	+
2	EL	1:16	+	+	+	+	+	ND	+	−	−
3	EL	1:128	+	+	+	−	+	+	−	−	−
4	1°	1:16	−	+	+	+	+	ND	+	+	+
5	2°	1:64	−	+	+	−	+	+	−	+	−
6	2°	1:32	+	+	+	+	+	ND	+	+	+
7	EL	1:32	+	+	+	−	+	+	+	−	−
8	1°	1:4	−	−	−	−	−	ND	−	−	−
9	1°	NR[c]	−	−	−	−	−	ND	−	−	−
10	2°	1:32	−	ND	+	−	+	+	−	+	−
11	EL	1:32	+	ND	+	+	+	ND	+	+	+
12	Normal	NR	ND	−	−	−	−	−	−	−	−

[a] Stage of syphilis: primary (1°), secondary (2°), and early latent (EL). Normal, control without syphilis.
[b] DF, dark-field microscopy; B, boiling method; S, low-spin separation method; SE, spin extraction method; AL, alkaline lysis extraction method; ND, not done.
[c] NR, nonreactive. Rapid plasma reagin was reactive (1:1), and the patient had a classical indurated chancre.

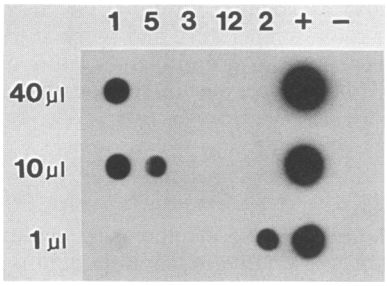

FIG. 1. Autoradiograph of syphilitic amniotic fluids processed for PCR by the alkaline lysis extraction method. Numbers in the top row indicate patient numbers shown in Table 2. + and −, positive and negative PCR reagent controls, respectively. Patient 12 had normal amniotic fluid. The volumes given at the left reflect the amounts of DNA solution subjected to PCR after alkaline lysis extraction of 100 µl of amniotic fluid.

which the boiling, low-spin separation, and alkaline lysis methods showed differing results; all four specimens tested positive by the spin extraction PCR method (Table 2, no. 3, 5, 7, and 10).

Various amounts (1, 10, or 40 µl) of the DNA extracted from amniotic fluid specimens were used in the alkaline lysis PCR (Table 2). Four different representative PCR results obtained with this strategy are shown in Fig. 1. Interestingly, increasing amounts of extracted DNA sometimes diminished rather than enhanced the sensitivity of the PCR assay (Fig. 1, lanes 2 and 5). The combined results for alkaline lysis extraction of the amniotic fluids revealed that 71% (5 of 7) for 1 µl, 57% (4 of 7) for 10 µl, and 43% (3 of 7) for 40 µl were positive relative to the numbers positive by RIT (Table 2).

Neonatal CSF and sera. The clinical features of 20 newborns of syphilitic mothers enrolled in this study are presented in Table 3; all infants underwent lumbar punctures and CSF examinations soon after birth. All but two of the CSF were subjected to RIT and PCR with both the boiling and low-spin separation methods (Table 4). Five specimens (no. 13 to 17) were positive by RIT, and three of these also were positive by PCR. The 13 remaining CSF were negative by both the RIT and the PCR (Table 4).

Six neonatal serum samples, five obtained at birth (Table 4, no. 16, 17, 18, 31, and 32) and one obtained during pregnancy by percutaneous umbilical blood sampling (data not shown), were positive by RIT. Four of these also were positive by PCR (boiling method) (Table 4; 16, 17, 31, and 32). Serum samples obtained at birth from the other 15 infants were not tested by RIT, but the PCR results were positive for five of these neonatal serum samples (Table 4, no. 13, 14, 15, 19, and 20); with the exception of infant 20, these neonates were symptomatic at birth (Table 3). The PCR results were negative for 12 serum samples from seronegative, healthy infants (data not shown).

Thirty-eight of the 40 CSF and serum specimens obtained at birth were processed for PCR by the boiling and low-spin separation methods, and 12 samples (3 CSF and 9 serum samples) were PCR positive by one or both methods (Table 4). All 12 of these were positive by PCR with the boiling method, but only 4 of these 12 were positive by the low-spin separation method (data not shown).

Four serum samples and CSF that were PCR negative by the boiling and low-spin separation methods but positive by RIT (Table 4, no. 14, 16, and 18; data not shown for one patient) were subjected to PCR with the alkaline lysis extraction protocol; they also were negative by this PCR protocol (data not shown).

TABLE 3. Clinical characteristics of syphilitic mothers and their neonates

Patient no.	Clinical characteristics of the following:					
	Mother		Neonate			
	Stage[a]	Serum VDRL	CL[b]	Serum VDRL	CSF[c]	CSF VDRL
13	2°	1:512	A	1:128	N	NR[d]
14	EL	1:64	A	1:128	N	1:1
15	EL	1:32	A	1:128	C	1:2
16	2°	1:128	N	1:16	N	NR
17	2°	1:32	A	1:32	P	NR
18	2°	1:32	N	1:2	C	NR
19	EL[e]	1:16	A	1:4	N	NR
20	1°	1:32	N	1:4	N	NR
21	EL	1:64	N	1:8	N	NR
22	EL	1:16	N	1:8	N	NR
23	EL	1:16	N	1:4	N	NR
24	EL	1:8	N	NR	N	NR
25	1°	1:8	N	1:1	N	NR
26	EL	1:32	N	1:1	N	NR
27	EL	1:16	N	1:8	P	NR
28	2°	1:32	N	1:4	N	NR
29	EL	1:256	A	1:64	N	NR
30	EL	1:16	N	1:16	P	1:1
31	2°	1:32	A	1:32	P	1:32
32	EL	1:4	A	1:128	C,P	1:4

[a] Stage of the disease as defined in Table 2, footnote *a*.
[b] CL, Clinical examination at birth, including examination for osteochondritis (A, abnormal; N, normal).
[c] CSF parameters: N, normal; C, elevated leukocyte levels for age; P, elevated protein concentration for age.
[d] NR, nonreactive.
[e] Treated during pregnancy.

Follow-up CSF and sera. If *T. pallidum* DNA persists in clinical specimens for appreciable periods following antimicrobial therapy, then positive PCR results for posttherapy specimens could misleadingly imply treatment failure. RIT, on the other hand, detects exclusively virulent organisms. To examine this, PCR analysis was performed on follow-up CSF and serum samples obtained 2 to 7 months after treatment from three patients whose pretherapy specimens were positive by RIT and/or by PCR (Table 4, no. 13, 15, and 17). All three were negative by both PCR and RIT. Representative positive and negative PCR results obtained with the boiling method on serum and CSF before and after treatment of patient 15 are shown in Fig. 2.

The 16 RIT-negative (13 at birth and 3 at follow-up) CSF all were negative by PCR (Table 4). Seven follow-up serum samples (Table 4) were also obtained 2 to 7 months after treatment from patients (no. 13 to 19) who had either a positive RIT or a positive PCR at birth (with CSF or serum). The PCR was negative for all seven cases, but the RIT was not performed.

Sensitivity and specificity. A compilation of the results for RIT and PCR applied to amniotic fluids, neonatal CSF, and neonatal sera is given in Table 5. Relative to the RIT, the sensitivities of the PCR for amniotic fluids, CSF, and sera were 100%, 60%, and 67%, respectively, with an overall sensitivity of 78%. For all specimens tested, the specificity was 100%.

TABLE 4. PCR detection of *T. pallidum* in CSF and serum of neonates at risk for congenital syphilis[a]

Patient no.	Results at birth				Results of follow-up test			
	CSF		Serum		CSF		Serum	
	RIT	PCR	RIT	PCR	RIT	PCR	RIT	PCR
13	+	+	ND	+	−	−	ND	−
14	+	−	ND	+	ND	ND	ND	−
15	+	+	ND	+	−	−	ND	−
16	+	−	+	+	ND	ND	ND	−
17	+	+	+	+	−	−	ND	−
18	−	−	+	−	ND	ND	ND	−
19	−	−	ND	+	ND	ND	ND	−
20	−	−	ND	+				
21	−	−	ND	−				
22	−	−	ND	−				
23	−	−	ND	−				
24	−	−	ND	−				
25	−	−	ND	−				
26	−	−	ND	−				
27	−	−	ND	−				
28	−	−	ND	−				
29	−	−	ND	−				
30	−	−	ND	−				
31	ND	ND	+	+				
32	ND	ND	+	+				

[a] Abbreviations are the same as in Tables 2 and 3. For PCR, all specimens were subjected to both the boiling and low-spin separation methods.

DISCUSSION

The objective of this study was to exploit the exquisite sensitivity and specificity of the PCR for the detection of low numbers of *T. pallidum* in clinical materials; we reasoned that this should be particularly relevant to the diagnosis of congenital syphilis, for which definitive diagnosis by direct detection of the organism is desirable but difficult to accomplish.

It is well recognized that contamination of reagents and specimens, introduced either during collection or upon sample processing, is a major cause of false-positive PCR results (32). The need to determine the presence of contamination underscores the importance of correlating PCR results to those obtained by using an independent method of high sensitivity and specificity. In the case of the noncultivatable pathogenic treponemes, RIT is the only technique currently available for such comparisons. We did not find any false positives among either the 21 RIT-negative samples or the 12

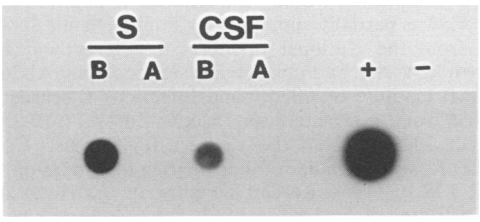

FIG. 2. Autoradiograph of follow-up PCR with serum and CSF from an infant previously shown by RIT and PCR to harbor *T. pallidum*. Samples were from patient 15, whose results are described in Tables 3 and 4. Samples were processed for PCR by the boiling method. PCR was performed before treatment (at birth) (B) and upon follow-up at 6 months after treatment (A). Serum (S) and CSF were assayed. + and −, positive and negative reagent controls, respectively.

TABLE 5. Combined comparison of PCR and RIT on amniotic fluids, neonatal CSF, and fetal or neonatal sera at birth and at follow-up

Sample	Test result	No. of samples per result of test		Sensitivity (%) of PCR relative to RIT	Specificity (%)
		RIT	PCR		
Amniotic fluid	+	7	7	100	
	−	5	5		100
CSF	+	5	3	60	
	−	16	16		100
Serum	+	6	4	67	
	−	0	12		100[a]
Total	+	18	14	78	
	−	21	33 (21 + 12[a])		100

[a] Inferred from the fact that sera were from normal subjects not infected with syphilis.

other normal serum samples, yielding a specificity of 100% and thus indicating the absence of contamination during specimen collection and processing. Although many of the follow-up specimens were not tested by RIT, all specimens which were RIT negative at follow-up also were negative by PCR when collected 2 to 7 months after treatment of the infant. Using PCR to test adult CSF, Hay et al. (21) reported the detection of *T. pallidum* in 53% of cases of late and tertiary syphilis. However, this figure is inordinately high compared with the findings by Lukehart et al. (34), who were unable to demonstrate the presence of *T. pallidum* by RIT in 18 CSF collected from patients with either early latent or late latent syphilis. Given the concern regarding the potential for PCR contamination, the absence of RIT confirmation by Hay et al. (21) precludes precise interpretation of their PCR results.

We demonstrated previously that under laboratory conditions, PCR is theoretically equivalent in sensitivity to the RIT (7) for detection of *T. pallidum*. In the present study, the PCR had a 100% sensitivity compared with the RIT when applied to amniotic fluid, a body fluid which typically contains relatively high numbers of *T. pallidum* (45, 46). The occurrence of significant numbers of treponemes in amniotic fluids appears to be more common for patients with secondary or later stages of the disease, inasmuch as amniotic fluids from two patients with primary syphilis failed to reveal *T. pallidum* either by RIT or by PCR. However, for serum and CSF, which are likely to contain fewer organisms (13, 34, 44), PCR was less sensitive than RIT. There are a number of factors which potentially diminished the sensitivity of the PCR when applied to CSF and sera. The large discrepancy in the volumes of specimens used for PCR and RIT (10 to 100 μl for PCR compared with 0.5 to 2 ml total for RIT) provides a plausible explanation for why the sensitivities of the PCR were not higher with CSF and sera. Also, the storage conditions for our specimens throughout the study could account for some false negativity by PCR compared with RIT, because some of our samples initially were not stored under optimal conditions (i.e., immediately frozen at −70°C); we have noted a drop in titers of treponemes, as assayed by PCR, when suspensions were stored for various periods at −20°C (unpublished observations).

Although noted by other investigators (4–6, 18–20, 22, 30, 40, 41), PCR inhibition exhibited by clinical materials still has not been given adequate attention; in this respect, significant limitations and challenges remain in attempting to

apply PCR as a diagnostic modality. Inhibition of PCR leading to false-negative results probably accounted for some of the lower PCR sensitivities obtained in our studies. We first encountered this phenomenon in former studies in which leukocytes or rabbit testicular tissue was added to suspensions of *T. pallidum* or *T. pallidum* DNA prior to PCR (7). Our present studies demonstrated that the inhibitory substances or activities often could be removed or destroyed but that different types of specimens required different methods of preparation for optimal PCR results. For example, the boiling and low-spin separation methods performed very well with amniotic fluids but to a lesser extent with a few CSF and sera, as did the alkaline lysis method. The fact that PCR results were routinely positive for amniotic fluids, typically rich in host cell debris (contributing to PCR inhibition) but also sometimes containing only few treponemes (i.e., dark-field negative), suggests that the boiling and low-spin separation methods indeed overcame PCR inhibition. However, the fact that some CSF and sera remained negative by PCR, despite several methods of sample preparation, indicates that inhibition of PCR continues to be problematic.

We sometimes circumvented PCR inhibition with amniotic fluids by using lesser quantities of DNA extracted by the alkaline lysis procedure. In several instances, PCR amplification occurred with 1 or 10 μl of DNA solution but not with 40 μl. With at least two specimens, it was surprising to find that PCR amplification occurred with 10 μl of the DNA mixture but not with 1 or 40 μl; this could be explained by the larger volume inhibiting *Taq* polymerase but the smaller volume lacking a complete copy of the 47-kDa antigen gene. In the case of sera, it also was found that PCR inhibition could sometimes occur when 20 μl of serum was used in the boiling procedure, which disappeared when only 10 μl of the sample was similarly processed. Thus, whenever feasible, consideration should be given to analyzing various amounts of extracted DNA from clinical specimens processed for PCR.

Despite the problems of applying PCR to clinical materials, there were a number of circumstances which substantiated the use of PCR in the diagnosis of congenital syphilis. Although five neonatal serum samples of infants born from mothers with untreated syphilis were positive by PCR but not tested by RIT at the time of specimen collection (at birth), four of those five infants had clinical and/or radiological findings (e.g., hepatosplenomegaly and/or osteochondritis or periostitis) compatible with the diagnosis of congenital syphilis. The fifth infant clearly was at significant risk for congenital syphilis. Although asymptomatic infection is common in cases of congenital syphilis, there is increasing concern that rising rates of maternal asymptomatic (incubating) syphilis (15) will exacerbate the problem of asymptomatic congenital syphilis diagnosis. This poses the challenge to continue to seek new molecular strategies for the diagnosis of congenital syphilis.

Another one of our cases is noteworthy with respect to the potential use of PCR in influencing the management of neonates with suspected congenital syphilis. During the pregnancy of patient 19, both amniotic fluid and fetal serum were RIT positive (PCR results were positive for amniotic fluid), thereby establishing that the fetus was infected at the time of diagnosis. The mother received two weekly intramuscular injections of penicillin G benzathine (2.4×10^6 U each) during pregnancy. Eleven weeks after treatment, the newborn at delivery showed mild hepatosplenomegaly and his serum was PCR positive. He thus was thought still to have congenital syphilis and was treated for 14 days with parenteral penicillin G. Although confirmatory RIT was not done with the serum, PCR used in this manner potentially can help address the issue of optimal treatment for congenital syphilis during pregnancy (10). The putative treatment failure also provides an impetus for performing much-needed studies on congenital syphilis treatment efficacy.

The results of our studies have allowed us to propose a strategy for using PCR in the diagnosis of congenital syphilis; amniotic fluids should be processed by the boiling and low-spin separation methods. For CSF and sera, in which often only very limited amounts of material are available, the simple boiling method with 10 and 20 μl should be performed first, followed by the low-spin separation method. Although we did not increase the overall sensitivity of PCR for any of the clinical specimens by using the more complex alkaline lysis method, alkaline lysis has been used by other investigators to overcome PCR inhibition (5, 19, 30) and could possibly yield better results if larger amounts of fluids could be processed.

The precise value of PCR in the diagnosis of congenital syphilis remains to be more fully evaluated. This would include performing a study in which the sensitivity of PCR can be prospectively evaluated relative to the RIT to maximize reliability of PCR by testing freshly obtained clinical materials. Results of PCR also should be compared with results from other test modalities, and thus a large prospective study combining clinical evaluation of the infant at birth by RIT, PCR, and detection of specific fetal IgM by Western blotting (14, 33, 42) is warranted. The PCR technology described in this study will be invaluable in such studies and will assist in obtaining important information concerning the reliabilities of new diagnostic strategies and also regarding the management of antepartum syphilis and congenital syphilis in the neonate. The PCR also should be used as a molecular probe to learn more about the pathogenesis of congenital syphilis; for example, it is feasible to use PCR to determine whether spirochetemia in infected infants is continuous and how long it lasts and what the relationship is between the presence of *T. pallidum* in serum and CSF. The clinical implications for the prognosis and treatment upon identification of *T. pallidum* in neonatal CSF are still unknown, but PCR also could represent a valuable means for the diagnosis and evaluation of congenital neurosyphilis.

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ADDENDUM IN PROOF

Neonatal CSF of patients no. 31 and 32 (Table 4) was found to be positive for *T. pallidum* by both PCR and RIT, making the sensitivity of PCR relative to RIT 71% for CSF, with an overall sensitivity of 80%.

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