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Blood Spot Screening and Confirmatory Tests for Syphilis Antibody

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We developed a blood spot test for syphilis antibody using enzyme-linked immunosorbent assay (ELISA) technology. Dried blood was eluted by buffered saline or, for a supplementary confirmatory test, by treponemal-antibody test diluent. Eluates were diluted in an absorption buffer (Calypte Biomedical, Berkeley, Calif.) and added to plate wells coated with cardiolipin antigen (ADI Diagnostics, Toronto, Ontario, Canada). The wells were washed and treated sequentially with an immunoglobulin G conjugate, buffer washes, and enzyme substrate. Substrate conversion was measured photometrically, and specimen reactivity was determined by reference to nonreactive controls. The optimum test protocol was established by tests of serum and plasma. The serum ELISA specificity with normal specimens was 98.9%. The sensitivity with sera from patients with undefined syphilis was 97.4%, that with sera from patients with documented primary and secondary disease was 100%, and that with sera from patients with early and late latent disease was 95.7%. The specificity of the spot test with donor blood was 94.2%, and its specificity with newborn blood was 94.9%. The sensitivity with 25 spots spiked with reactive sera was 96%. The seroprevalence rates for parturient women in one hospital were 6.01% according to spot tests of sera from 599 newborns and 6.81% according to Rapid Plasma Reagin tests of 499 maternal serum specimens. Seventy percent of infants born to 50 seropositive women were reactive by either the newborn spot or the Rapid Plasma Reagin serum test. The results show that blood spots may be used in seroprevalence or serodiagnostic studies, especially to identify women who are infected or to identify possible cases of congenital infection. The test provides for studies of children and adults when routine venipuncture and serum handling and storage are problematic.

The incidence of infectious syphilis in the United States has increased sharply in recent years (16, 24, 26). Increases have been reported for cities, in association with human immunodeficiency virus (HIV) infection, and among opiate and cocaine drug abusers, prostitutes, and newborns (6, 7, 12, 14, 22, 23). Public health efforts to contain this outbreak by identifying the prevalence and distribution of infection and by diagnosing infectious disease early are based primarily on serologic tests (4, 5, 17, 26).

Protocols for serologic tests for syphilis dictate nontreponemal cardiolipin antigen screening tests, supplemented when indicated by confirmatory treponemal-antibody tests (10, 13). Nontreponemal-antibody assays using enzyme-linked immunosorbent assay (ELISA) serochemistry have been described elsewhere (20, 21, 28). These new techniques show a sensitivity and specificity comparable to those of flocculation procedures (26a, 28). In addition, these assays offer the distinct advantages of instrument-objective test readings and formats suitable for manual or automated screening of large numbers of specimens.

The increase in infectious syphilis, particularly in congenital disease, and the application of ELISA technology to screening tests suggest the use of ELISA for detecting nontreponemal antibody in dried blood spots. Blood spots have been found comparable to serum or plasma in studies of a number of infectious disorders: rubella, hepatitis, HIV disease, and most recently, yaws (1, 9, 27). Moreover, on the basis of detection of passively transferred antibody in blood spots obtained from newborns, HIV ELISAs confirmed by Western blot (immunoblot) assays indicate the prevalence of HIV in childbearing women and estimate the number of infected children in the general population (8, 11, 19).

A spot test for syphilis could be useful for studies of syphilis in childbearing women and could identify infants who may be infected (26b). The test could be used for young children, the elderly, or others, such as intravenous drug abusers, for whom venipuncture may be problematic. Finally, it could be useful when low-cost specimen collection and transport are imperative.

We developed a nontreponemal-antibody ELISA for screening blood spots for syphilis and established a protocol for confirming results by a treponemal test of the same specimen. This report describes the reagents and procedures and compares results obtained with blood spots with those obtained by standard tests of serum and plasma.

MATERIALS AND METHODS

ELISA protocol. The optimum ELISA protocol was established by repeated trials of reagents, reagent concentrations, reaction times, and absorbance cutoff values with sera characterized by standard cardiolipin and treponemal-antibody tests.

The test was conducted on phosphate-buffered saline (PBS, pH 7.2)-rinsed 96-well polystyrene plates with cardiolipin (0.0006%)-, lecithin (0.0042%)-, and cholesterol (0.09%)-coated wells (Visuwell; ADI Diagnostics, Toronto,

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Ontario, Canada). Serum or plasma at an initial dilution of 1/20 in PBS (pH 7.2), or fluid eluted from dried blood spots at an equivalent concentration, is suitable for testing. Twenty microliters of diluted specimen was mixed with 30 μl of 1.6× E-1 dilution buffer (Calypte Biomedical, Berkeley, Calif.) for a final dilution of 1/50 in the test plate well and incubated for 60 min at room temperature (RT). Each plate was washed six times (two cycles of three washes each) with 1.275% saline in an automated plate washer (Du Pont, Wilmington, Del.). Fifty microliters of an optimum concentration of horseradish peroxidase-conjugated F $(ab')_2$ rabbit anti-human immunoglobulin G (DAKO, Carpinteria, Calif.) in PBS containing 1% bovine serum albumin was added, and the plates were incubated for 60 min at RT. After a second series of six washes, 50 μl of the substrate 2,2'-azino-di-(3-ethyl)benzothiazoline-6-sulfonic acid) (ABTS; 50 mg/100 ml in buffer containing disodium hydrogen peroxide [Boehringer Mannheim, Indianapolis, Ind.]) was added. After 45 min of incubation at RT, the plates were swirled to ensure even distribution of converted substrate, and the A_{414} was read versus an air blank in an automated plate reader (Du Pont).

Blood spot test. Specimens for neonatal screening (Guthrie spots) for inherited metabolic disorders consisted of blood on 1/2-in. (1 in. = 2.54 cm) targets on special Schleicher & Schuell (Keene, N.H.) no. 903 filter paper (8, 15). The blood was collected according to a National Committee for Clinical Laboratory Standards protocol (15) and dried for at least 3 h at RT before laboratory testing. For the spot test, 1/4-in. disks containing 5 μl of serum (12.5 μl of whole blood) were punched out of the filter paper from neonatal or adult specimens. The disks were soaked in 100 μl of PBS or absorbing diluent (see below) for 18 h at 5°C on a platform shaker (New Brunswick Scientific model 2R) at 75 oscillations per min. Eluates contained the equivalent of a 1/20 serum dilution. For the ELISA only, 100 μl of PBS (pH 7.2) was used for elution; for the ELISA and the confirmatory treponemal test, 100 μl of absorbing diluent (MHA-TP; Miles Diagnostics, Elkhart, Ind.) was used. Absorbing Diluent remaining from spots found reactive in ELISA was examined in the standard MHA-TP protocol. Alternatively, a second 1/4-in. sample may be punched for a confirmatory test.

Test controls. Each test plate included two blank wells with 50 μl of PBS or MHA-TP diluent only, four negative-reference standard wells with nonreactive serum or blood spot eluates, and two positive-reference standard wells with mid-range reactive serum or blood spot eluates.

Standard tests for syphilis. Serum and plasma specimens were tested by standard qualitative and (when necessary) quantitative Automated Reagin Test or Rapid Plasma Reagin (RPR) card flocculation procedures (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Reagin test-reactive specimens were reexamined by a fluorescent treponemal-antibody (FTA-ABS; Scimedx, Denville, N.J.) test or MHA-TP test. Previously published Centers for Disease Control procedures were used for all tests (13) and all runs included controls and reading standards from test manufacturers and from the Wadsworth Center for Laboratories and Research.

Test specimens. All patient identifiers were removed from all study sera or blood spots before experimental tests for syphilis.

(i) **Serum.** Aliquots were obtained from serum specimens after completion of serodiagnostic tests at the Wadsworth Center and from plasma specimens after completion of blood donor tests at the American Red Cross Greater Upstate New York Blood Services Center. The specimens were from four groups: reagin and treponemal-antibody test-reactive sera (n = 309), RPR-nonreactive blood donors (n = 92), sera from patients with documented stages of syphilis (n = 54), and biologic false-positive (reagin test-reactive and treponemal test-nonreactive) sera (n = 43). The specimens were tested after storage at 5°C for not more than 6 days or after storage at −20 or −70°C.

(ii) **Blood spots.** Whole blood in EDTA anticoagulant was obtained from individual donors within 2 days of collection. To determine the spot test's specificity and to establish normal background values, nonreactive spots were prepared by adding 50 μl of whole blood from each of 411 RPR-nonreactive donors to the 1/2-in. targets of filter paper cards and air drying the spots at RT for 24 to 72 h. To test relative sensitivity, a measured volume of plasma was removed from type O normal donor blood and replaced with an equal volume of serum from one of 25 reagin test-reactive (titer, 4 to 32), treponemal test-reactive specimens. After mixing, blood spots were prepared as described above for nonreactive spots. The final RPR titer of the spiked blood was subsequently established by a test of serum recovered from the donor blood-reactive serum mixture.

Surplus newborn-blood spot specimens were obtained from the Wadsworth Center after all screening tests had been completed. Specimens included spots obtained from (i) 15 RPR-nonreactive and 35 RPR-reactive infants born to confirmed seropositive women, (ii) 219 infants born in geographic regions designated low-prevalence areas on the basis of the epidemiology of early infectious and congenital syphilis in New York State (18), and (iii) infants (599 blood spots) born within a defined period in an urban hospital with a relatively high prevalence of congenital syphilis. For comparison with the last collection, maternal serum was obtained from the same hospital for 499 births occurring in the same period.

Newborn-blood spots retained at room temperature were tested within 24 days of collection. Spots held for longer times and control spots were stored for up to 90 days at 5°C, and for longer times at −20°C, in gas-impermeable plastic bags (Bitran Saranex-Series S; American Scientific Products, Pittsburgh, Pa.) with desiccant (Sorb-it; United Desiccants-Gates, Camden, N.J.) (9).

Reproducibility. The precision of the spot test was measured by tests of mid-range reactive spots prepared from blood with an RPR titer of 16 and nonreactive spots from an RPR-nonreactive donor. Triplicate spots from each reference collection were included in each of 12 test runs conducted over 30 days.

Stability of stored spots. Tests were conducted on spots after storage with desiccant at −20°C for 6 months. Results of tests on 25 reactive (described in "Blood spots" above) and 50 donor spots were compared with those of other samples from the same filter paper tested within 10 days of preparation. The ratios (absorbance/cutoff) obtained with spots that had been stored frozen were compared with those of the freshly prepared spots.

RESULTS

ELISA serochemistry. Comparative results of tests of normal donor sera and sera submitted for and found reactive in serologic tests for syphilis are shown in Fig. 1. The most accurate ELISA separation of the specimens into nonreactive and reactive groups occurred at 0.130 absorbance units.

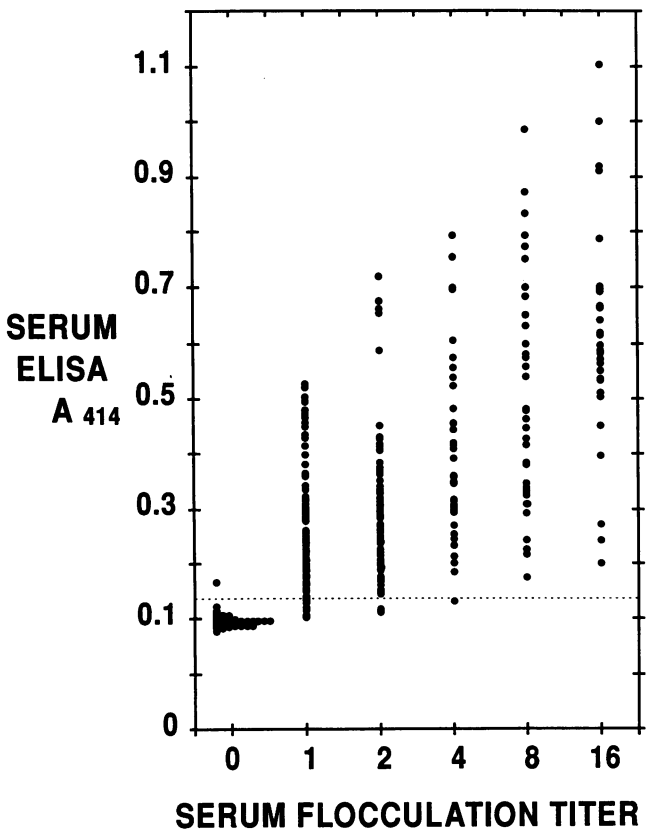

FIG. 1. Comparison of serum ELISA absorbance values with qualitative and quantitative flocculation test results for serum specimens. Results for 92 RPR test-nonreactive (titer, 0) donor serum specimens and for 309 Automated Reagin Test-reactive (titer, 1 to 16), FTA-ABS-reactive diagnostic serum specimens are shown. The dashed line drawn at 0.130 absorbance units indicates the test cutoff at 1.35 times the mean nonreactive-control value (0.096). Sera above the line were scored as reactive in the ELISA test.

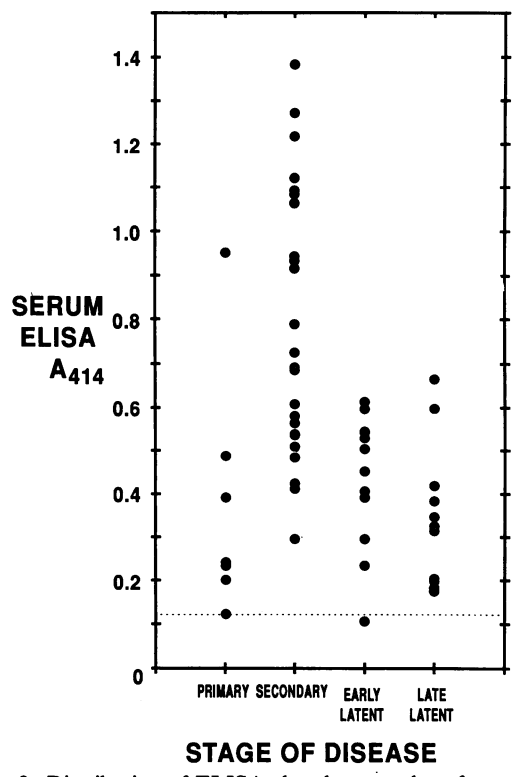

FIG. 2. Distribution of ELISA absorbance values for sera from 54 patients with documented stages of syphilis. The dashed line at 0.123 absorbance units indicates the test cutoff at 1.35 times the mean nonreactive-serum control value (0.091).

This cutoff, at 1.35 times the mean nonreactive control absorbance, resulted in correct identification of 91 of 92 donors as nonreactive, for a relative specificity of 98.9%. The single donor specimen above the cutoff was MHA-TP nonreactive. The relative sensitivity of the ELISA with sera was 97.4%, with the absorbances of 301 of the 309 specimens above the calculated cutoff. As shown in Fig. 1, there was a positive relationship between the titer and ELISA absorbance. Five of the eight serum samples with absorbance values below the cutoff were minimally reactive (titer 1) in the flocculation test, two had a titer of 2, and one had a titer of 4. A similar distribution of results for serum specimens from documented stages of syphilis is shown in Fig. 2. In this comparison, the absorbances of 53 of 54 specimens were greater than the ELISA cutoff. The sensitivity with primary and secondary sera was 100% (all 31 specimens had absorbance values above the cutoff). A single early latent specimen with a titer of 2 had an absorbance less than the cutoff. The sensitivity with early and late latent sera was therefore 95.7% (22 of 23 specimens had absorbances above the cutoff).

ELISAs of biologic false-positive sera (flocculation test reactive titer of 1 to 16 and FTA-ABS nonreactive) resulted in 40 of 43 specimens (93%) being reactive. The three specimens that were nonreactive in the ELISA had flocculation test titers of 1.

Blood spot serologic results. The absorbance findings for presumed normal spots prepared from blood from donors or newborns are shown in Table 1. The data are derived from single tests of three donor groups (n = 199, 12, and 200; total = 411), and of five newborn groups (n = 67, 25, 25, 34, and 68; total = 219). Cutoffs for each of the spot tests were calculated as described above for tests of serum, except that the nonreactive standards were four donor blood spots (data not shown). The specificity of the spot test according to the adopted cutoff formula with these specimens was 94.2% for adult donors, with 387 of 411 specimens found nonreactive, and 94.9% (208 nonreactive specimens out of a total of 219) for newborns. None of the specimens with absorbance values above the cutoff were MHA-TP reactive.

The distribution of results of single tests of spots prepared from spiked whole blood and quantitative flocculation tests of the plasma recovered from the spiked blood is shown in Fig. 3. The sensitivity of the spot test with this set of

TABLE 1. Spot test absorbance values for presumed normal blood spots

Source of Spot	No. of specimens	Absorbance		
		Mean	SD	Range
Adult donors	411	0.167	0.031	0.108–0.329
Newborn infants	219	0.171	0.030	0.108–0.293

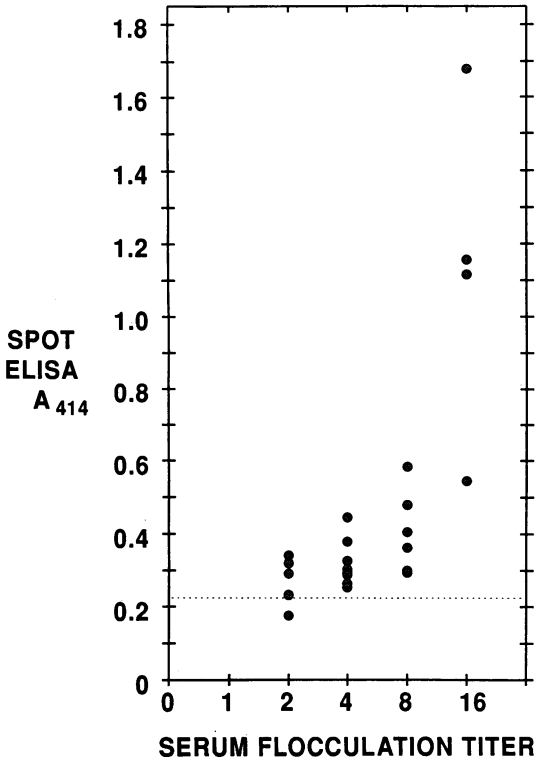

FIG. 3. Spot test absorbance values for spots prepared from 25 blood specimens with the final RPR titers shown. The dashed line at 0.224 absorbance units indicates the spot test cutoff at 1.35 times the mean absorbance of the nonreactive blood spot control (0.165).

prepared specimens was 96% (24 of 25 specimens had A_{414}s above the cutoff).

The seropositivity of spot tests for infants born to 50 confirmed seropositive women was comparable to that of RPR tests of infant serum. Twenty-eight infants were reactive in both the spot and RPR serum tests, 14 others were reactive only in the spot (n = 7) or RPR (n = 7) test, and 8 were nonreactive in both tests. Thirty-two (91.4%) of the 35 reactive spots were MHA-TP reactive.

Treponemal test confirmation shows excellent agreement (seropositivity rate, 6.01 versus 6.81%) between spot tests of blood from newborns and RPR tests of unmatched parturient maternal serum (Table 2). In this study, 76.6% (36 of 47) of reactive newborn-blood spots and 85% (34 of 40) of RPR-reactive maternal serum samples were also reactive in the MHA-TP test.

The reproducibility of the spot test is high, as shown by

TABLE 2. Prevalence of syphilis antibody in women giving birth in an urban hospital during a defined period

Specimen source	No. of specimens	No. of specimens reactive[a] in:			Prevalence (%)
		RPR test	ELISA	MHA-TP test	
Maternal serum	499	40		34	6.81
Newborn-blood spot	599		47	36	6.01

[a] Maternal serum samples were tested by the RPR test. Unmatched newborn-blood spots were tested by the ELISA. Sera reactive in the RPR test and spots reactive in the ELISA were tested by the MHA-TP test.

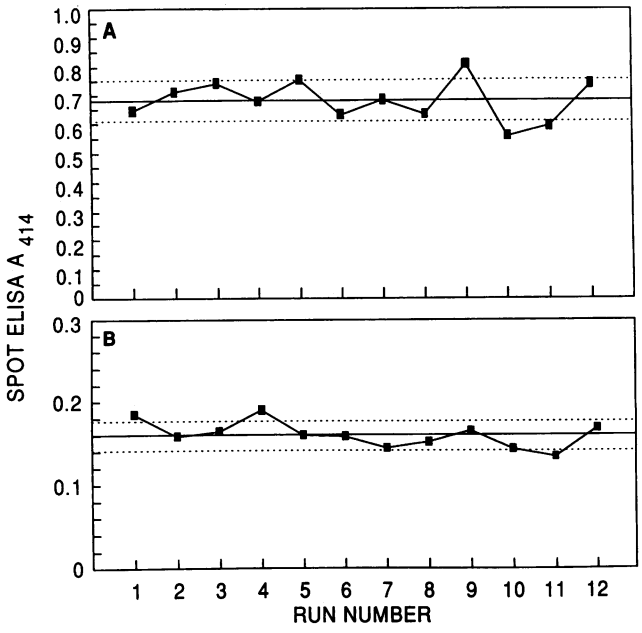

FIG. 4. Reproducibility of the spot test with reference blood spots. (A) Means of three absorbance values for each of 12 individual runs with mid-range reactive (RPR titer, 16) spots. The solid line drawn at 0.682 absorbance units represents the mean value for all 12 runs, and the dotted lines represent ±1 SD (0.072) from the mean. (B) Mean and SD values of nonreactive blood spots for the same runs (mean = 0.160, SD = 0.016).

the lack of scatter of the control values plotted in Fig. 4. The mid-range reactive- and nonreactive-control values did not exceed ±2 standard deviations (SD) of their means; the reactive-control values were within ±1 SD of the mean for 8 of the 12 runs, and the nonreactive-control values were within ±1 SD of the mean for 9 of the 12 runs.

Results obtained by retesting specimens after 6 months of storage at −20°C were comparable to those found after storage for 10 days at 5°C. There were no qualitative differences; that is, spots made with blood supplemented with known reactive sera were reactive, while donor spots were nonreactive. The means of absorbance values obtained by comparison of 25 reactive spots were 0.485 after 6 months of storage and 0.525 after 10 days of storage. For the 50 nonreactive donor spots, the corresponding values were 0.179 and 0.171.

DISCUSSION

It was essential, in developing the spot test, to separate the unique high background inherent in dried blood specimens from the reactivity specific to low antibody concentrations. Techniques used in other spot tests include detergents to suspend immunoglobulin aggregates in elution fluids and thus reduce nonspecific adsorption to test plates and prior addition of proteins such as bovine serum albumin or fetal calf serum to block plate surfaces not occupied by antigens. These measures are clearly successful with tests using protein antigens. Detergents, however, solubilize and detach cardiolipin-lecithin-cholesterol (CLC) antigen complexes from plates, and proteins are of limited effectiveness in blocking spaces among the adsorbed CLC. The spot test for syphilis uses (i) an optimum dilution of elution fluid to reduce the concentration of blood substances; (ii) E-1 dilution buffer

to absorb nonspecific substances, most particularly aggregated or denatured immunoglobulin (the result, reduced nonspecific reactivity, is comparable to that obtained in FTA-ABS tests with sorbent or in MHA-TP tests with absorbing diluent); and (iii) an affinity-purified $F(ab')_2$ conjugate to obviate complexing with Fc receptors present on some fragments of lysed erythrocytes and leukocytes in the specimen. These treatments combine to reduce the elevated background and minimize false-positive results. As a consequence, the test is capable of detecting a minimum of antibody in the small volume contained in a blood spot. Absorbance values of specimens with minimal antibody concentrations (titer of 1 to 4) show clear separation from values obtained with nonreactive specimens. As reported above, the overall specificity with blood spots exceeded 94%.

The sensitivity of the ELISA procedure with serum is indicated by the comparison of absorbance findings with quantitative flocculation titers. The absorbance values are roughly proportional to the flocculation titers. As indicated, the test detects antibody in each of the stages of syphilis; higher absorbance values matched higher flocculation titers, and as expected, higher absorbance values were found with sera from patients with secondary syphilis. It may be assumed that similar findings would be obtained by tests of blood spots from patients in different stages of disease.

The fact that 93% of the biologic false-positive sera were also reactive in the ELISA suggests that CLC antigenic components are correctly oriented on the polystyrene plates and that antibody detected by the ELISA test is qualitatively similar to that detected by flocculation procedures. The ELISA chemistry may obviate prozone reactions, in which specimens with high concentrations of nontreponemal antibody fail to react in flocculation tests (3, 13). Blood spots with high antibody concentrations, as may be found in congenital syphilis, should react in the ELISA, thus avoiding false-negative findings. Spot tests of blood from newborns detect passively transferred maternal antibody, and additional tests and clinical studies are necessary to establish a diagnosis of congenital disease.

The MHA-TP test protocol is effective in confirmation of spot test results. The *Treponema pallidum*-sensitized cell-settling patterns of reactive and nonreactive blood spots exactly match those obtained with serum. No nonspecific MHA-TP reactions, that is, agglutinations of unsensitized cells with spot elution fluid, were seen in this study. Earlier workers using an experimental *T. pallidum* hemagglutination procedure as a spot screening test found 3.8% of the reactive tests to be false positive (25). The difference in findings may be due in part to the greater specificity of our two-test protocol. Centers for Disease Control recommendations reserve treponemal-antigen tests for confirmation of CLC test findings (13). Thus, the two-test protocol preserves the specificity of the MHA-TP procedure; it may also be more economical, and in early disease more sensitive, than the MHA-TP test alone.

Quantitative nontreponemal test results are important in determining the efficacy of treatment and may be essential in detecting relapse or reinfection in patients previously found to be MHA-TP reactive. Additional studies are necessary to determine whether blood spot absorbance may substitute for serum flocculation titer in describing antibody concentration.

There are a number of advantages to the spot test protocol. A phlebotomist is not required for specimen collection. Simple, inexpensive devices may be used for collecting specimens, spots may be transported at low cost, and large numbers of specimens may be stored in very little refrigerator or freezer space. Our findings are consistent with those of other studies (9) which indicate that, under laboratory conditions, antibodies in stored blood spots are stable. Blood spots have been suggested as an excellent alternative to serum for HIV antibody tests when specimens are collected and stored under adverse tropical conditions (2). It is important to add that eliminating blood tubes eliminates spill and aerosol dangers attributable to specimen handling operations such as uncapping, centrifuging, and separating or transferring serum. Finally, there is a marked reduction in the volume and weight of hazardous waste, whose disposal is costly.

The spot test protocol described in this report is suitable for larger-scale evaluation studies to establish its clinical and laboratory usefulness. The test presents an opportunity for seroprevalence and serodiagnostic programs. For seroprevalence, results of newborn screening tests would be especially helpful in estimating infection and disease in women and children, in describing demographic patterns associated with perinatal transmission, and in developing prevention and health care services. For serodiagnosis, screening tests of infants would help establish maternal infection and thus potential congenital disease, thereby reducing syphilis morbidity and health care costs by direct, early intervention. Finally, the spot test may make specimen collection and transport efficient and economical for special test programs.

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