Blood Spot Screening and Confirmatory Tests for Syphilis Antibody

ROY STEVENS,1,2,* KENNETH PASS,1,2 STEVEN FULLER,3 ANDREW WIZNIA,4 LAWRENCE NOBLE,4 SALVATORE DUVA,1 and MICHAEL NEAL3

Wadsworth Center for Laboratories and Research and School of Public Health, State University of New York at Albany,2 Albany, New York 12201; ADI Diagnostics, Toronto, Ontario M9W 4Z7 Canada; and Bronx Lebanon Hospital, Bronx, New York 10468

Received 20 March 1992/Accepted 19 June 1992

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 intravenous 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) serochimerstry 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 (Visiwell; ADI Diagnostics, Toronto,
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, Carpenteria, 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 A414 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 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 glass vials or 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 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 59 nonreactive 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.
men with greater reactive titer of Reagin values above the cutoff. The sensitivity was 97.4%, was in 40 of 43 specimens (22% of 195.7%). The single cutoff, from documented floculation the positive test, was calculated as shown in Fig. 1. The relative sensitivity of the ELISA with sera was 97.4%, with the absorbances of 31 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 floculation 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 (floculation 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 floculation test titers of 1.

**Blood spot serologic results.** The absorbance findings for presumed normal spots prepared from blood 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 floculation 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**

<table>
<thead>
<tr>
<th>Source of Spot</th>
<th>No. of specimens</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Adult donors</td>
<td>411</td>
<td>0.167</td>
</tr>
<tr>
<td>Newborn infants</td>
<td>219</td>
<td>0.171</td>
</tr>
</tbody>
</table>
prepared specimens was 96% (24 of 25 specimens had $A_{414}$ 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 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 antibodies. 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 aggre-
gated 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 con-
jugate 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 conse-
quence, 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 as-
sumed 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 anti-
body fail to react in flocculation tests (3, 13). Blood spots
with high antibody concentrations, as may be found in
genital syphilis, should react in the ELISA, thus avoiding
false-negative findings. Spot tests of blood from newborns
detect passively transferred maternal antibody, and addi-
tional 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
de 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 concentra-
tion.

There are a number of advantages to the spot test proto-
col. 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 refriger-
ator 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 col-
lected 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 opera-
tions 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 seropre-
valence, results of newborn screening tests would be espe-
cially 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 mor-
bidity 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.

ACKNOWLEDGMENTS

We thank Tina Charbonneau of the Wadsworth Center and the
staff of the American Red Cross Greater Upstate New York Blood
Services Center for the collection and processing of surplus samples
of anonymous-donor blood.

REFERENCES

1. Backhouse, J. L., M. H. Lee, S. I. Nesteroff, B. J. Hudson, and
Microbiol. 30:561–563.
2. Behets, F., M. Kashamuka, M. Pappaoianou, T. A. Green, R. W.
1992. Stability of human immunodeficiency virus type 1 anti-
obodies in whole blood dried on filter paper and stored under
various tropical conditions in Kinshasa, Zaire. J. Clin. Micro-
biol. 30:1179–1182.
False negative tests for syphilis in pregnant women. N. Engl. J.
5. Centers for Disease Control. 1988. Recommendations for diag-
osing and treating syphilis in HIV-infected patients. Morbid.
6. Centers for Disease Control. 1988. Relationship of syphilis to
drug use and prostitution—Connecticut and Philadelphia, Penn-
829.
immunodeficiency virus antibody in dried-blood specimens col-
lected on filter paper from neonates, p. 1–33. Centers for
Disease Control, Atlanta.
A quality assurance program for human immunodeficiency virus
seropositivity screening of dried-blood spot specimens. Infect.

Downloaded from http://jcm.asm.org/ on August 27, 2017 by guest


