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Evaluation of the Hemagglutination Treponemal Test for Syphilis

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The sensitivity and specificity of the hemagglutination treponemal test for syphilis (HATTS) was compared with the fluorescent treponemal antibody-absorption test (FTA-ABS) with 491 sera. Medical histories were obtained for 153 patients with seroreactivity in either treponemal test. Overall correlation with patient history was 96.7% for the FTA-ABS and 93.9% for the HATTS. False-negative HATTS occurred in primary, late-latent (>2 years), and treated syphilis. The reproducibility of the HATTS and FTA-ABS is equivalent. HATTS is an acceptable treponemal confirmatory test for syphilis, but the FTA-ABS should be used to resolve diagnostic discrepancies.

The hemagglutination treponemal test for syphilis (HATTS), as developed and marketed by Difco Laboratories, was described and evaluated by Wentworth et al. in 1978 (7). Peter et al. (5) have compared the HATTS with the fluorescent treponemal antibody-absorption test (FTA-ABS) (6) and concluded that it was preferable to the FTA-ABS as a routine treponemal test for confirming sera found reactive by nontreponemal tests.

Larsen et al. (3) have evaluated specificity, sensitivity, and reliability of HATTS and concluded that the FTA-ABS, HATTS, and microhemagglutination assay for *Treponemal pallidum* tests were comparable, although both hemagglutination tests lacked sensitivity in sera from patients with primary syphilis. HATTS was granted provisional status as a serological test for syphilis as a result of this study.

The present study was undertaken to provide comparative data between the HATTS and FTA-ABS which are used as confirmatory treponemal tests for sera found reactive by nontreponemal tests by public health laboratories or for sera referred by physicians to resolve diagnostic problems. We examined the sensitivity, specificity, and reliability of the HATTS and report the short- and long-term intralaboratory reproducibility for serologists trained to perform and interpret hemagglutination tests and the interlaboratory reproducibility for serologists from the Centers for Disease Control (CDC). A similar study on sera from hospital patients was simultaneously undertaken by Friedly et al. (1).

MATERIALS AND METHODS

Sera. Whole clotted blood and serum specimens for diagnostic or prenatal syphilis serology were referred to the Hygienic Laboratory, University of Iowa, from physicians throughout Iowa. All sera were tested the day they were received in the laboratory. A total of 491 sera were included in the study. Of these, 273 were reactive by the Venereal Disease Research Laboratory (VDRL) slide test (6), and 153 were reactive by either treponemal test.

Reproducibility. The sera for the reproducibility sets were taken from six pools prepared by Difco Laboratories and designated A through F. The pools were prepared so that only pool A was reactive by the VDRL slide test, whereas pools A through D were reactive by both the FTA-ABS and HATTS. Pools E and F were nonreactive in both treponemal tests. The pools were divided into 0.5-ml samples, sealed in glass ampoules, coded numerically, and frozen as replicate sets of 60 specimens each. Sera in each set were selected to provide representative reactivities for both short- and long-term reproducibility studies consisting of 30 sera each. Sets of sera for the reproducibility study were shipped to CDC and the University Hygienic Laboratory on dry ice for independent testing to determine interlaboratory variation. These sera were thawed the day before testing and submitted to the laboratory as "blind" fictitious patient specimens. For short-term reproducibility, six sera were tested daily for 5 consecutive days. Long-term reproducibility was determined by testing six sera weekly for 5 consecutive weeks.

Serological testing. All sera were tested by the VDRL slide screening test with commercial reagents and by standard techniques (6). All sera demonstrating any degree of reactivity were retested by the VDRL slide quantitative test (6).

The FTA-ABS was performed by the standard procedure (6) and the HATTS was performed according to the instructions supplied by the manufacturer. Sera to be tested were heated for 30 min at 56°C (2) and then diluted 1:16 in test diluent containing 0.5 ml of HATTS test diluent dye. Next, 25 μl of each serum sample was placed in duplicate wells of a microtitration tray. Sensitized erythrocytes were added to one well, and unsensitized erythrocytes were placed in the second well. The final dilution of serum plus cells was 1:80. Plates were incubated undisturbed for 1 h at 26 ± 3°C. Readings of HATTS results were based on the appearance of the hemagglutination pattern. Sera with readings of 1+ through 4+ were considered reactive. Sera read as ± were retested. Sera read twice as ± and negative sera were considered nonreactive. Sera reacting with unsensitized control erythrocytes were considered unsatisfactory for HATTS.

The serologists performing and interpreting the tests were experienced with the VDRL and FTA-ABS tests and were trained in the performance and interpretation of the HATTS by technical personnel from Difco Laboratories before the study commenced. Two serologists, one assigned to each test, performed and interpreted the FTA-ABS and HATTS throughout the study to ensure continuity and independence of readings. Controls and quality control parameters performed were in accordance with standard recommendations. The HATTS kits for the study were all from Difco lot number 704470. Serum dilutions were prepared with a Hamilton digital diluter.

Histories. A questionnaire was sent to the physician who

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TABLE 1. Results of the FTA-ABS compared with patient history of syphilis

Patient history	No. with the following FTA-ABS results	
	Reactive	Nonreactive
Syphilis	121	2
Not syphilis	14	354

TABLE 2. Results of the HATTS compared with patient history of syphilis

Patient history	No. with the following HATTS results	
	Reactive	Nonreactive
Syphilis	112	11
Not syphilis	19	349

TABLE 3. Results of VDRL, FTA-ABS, and HATTS tests for stages of disease determined by patient history

Patient history	No. of sera tested	No. with the indicated results by the following tests[a]:							
		VDRL			FTA-ABS			HATTS	
		R	WR	N	R	B	N	R	N
Primary syphilis	3	3	0	0	3	0	0	2	1
Secondary syphilis	12	12	0	0	12	0	0	12	0
Early-latent syphilis (<1 yr)	8	6	2	0	8	0	0	8	0
Early-latent syphilis (<1–2 yr)	5	1	2	2	5	0	0	5	0
Late-latent syphilis (>2 yr)	18	10	8	0	17	1	0	13	5
Late-latent syphilis (cardiovascular)	1	0	1	0	1	0	0	1	0
Late-latent syphilis (neurosyphilis)	7	6	1	0	7	0	0	7	0
Congenital syphilis	2	1	1	0	2	0	0	2	0
Maternal antibody	3	1	1	1	3	0	0	2	1
Syphilis (stage undetermined)	64	17	34	13	63	1	0	60	4
Not syphilis	27	8	11	8	12	4	11	18	9
Other disease	3	0	3	0	2	1	0	1	2

[a] R, Reactive; WR, weakly reactive; N, nonreactive; B, borderline.

TABLE 4. Method for comparison of history of syphilis and treponemal tests[a]

Patient history	Results by the treponemal test		
	Reactive	Nonreactive	Total
Syphilis	A	B	T1
Not syphilis	C	D	T2
Total	T3	T4	N

[a] Abbreviations: T, total; A/T1, sensitivity; A/T3, true positive; D/T2, specificity; D/T4, true negative; C/T3, relative false positive; C/T2, absolute false positive; B/T4, relative false negative; B/T1, absolute false negative; (A + D)/N, reliability.

TABLE 5. Comparative summary of FTA-ABS and HATTS results

Analysis[a]	Test result/total (%) for the following tests:	
	FTA-ABS	HATTS
Sensitivity	121/123 (98.4)	112/123 (91.1)
Specificity	354/368 (96.2)	349/368 (94.8)
Reliability	475/491 (96.7)	461/491 (93.9)
True positivity	121/135 (89.6)	112/131 (85.5)
True negativity	354/356 (99.4)	349/360 (96.9)
Relative false positivity	14/135 (10.4)	19/131 (14.5)
Relative false negativity	2/356 (0.6)	11/360 (3.1)
Absolute false positivity	14/368 (3.8)	19/368 (5.2)
Absolute false negativity	2/123 (1.6)	11/123 (8.9)

[a] Method used to analyze data presented in Table 4.

TABLE 6. Reproducibility study results for tests performed by Difco Laboratories, CDC, and the University Hygienic Laboratory[a]

Serum pool	Difco Laboratories										CDC													
	VDRL		FTA-ABS				HATTS				RPR				FTA-ABS					HATTS					
	N	1:8	N	1+	3+	4+	N	1+	2+	4+	N	1:1	1:2	1:32	N	1+	2+	3+	4+	N	±	1+	2+	3+	4+
A		1				1				1				1					2						3
B	1					1			1				1					2					2	1	
C	1			1				1				1				1	1				1	1	1		
D	1				1			1				1						1	1				2	1	
E	1		1				1				1				2					3					
F	1		1				1				1				2					3					

[a] Abbreviations: N, nonreactive; WR, weakly reactive.

submitted the specimen whenever a serum tested weakly reactive or greater. The questionnaire asked the physician to determine whether the patient had syphilis and, if infected, to indicate the stage of the disease. Physicians were asked whether reinfection had occurred, whether treatment was administered (including dates), and whether other diseases or circumstances were present which might affect results of treponemal tests. Questionnaires were compared with state health department records to confirm the stage of disease. The histories were used to determine the sensitivity, specificity, and reliability of the treponemal tests.

Statistical analysis. Significance was determined from the McNemar test for correlated proportions (4). Reproducibilities are presented as percentages of true values determined from patient history.

RESULTS

Of the 491 specimens included in this study, 273 were reactive by the VDRL test. When all specimens were examined by FTA-ABS and HATTS, 153 sera were found to be reactive by either treponemal test. Histories were obtained for patients reactive by either treponemal test, which identified 123 cases of syphilis and 30 cases of other diseases or no disease. A total of 338 specimens were nonreactive by both treponemal tests.

When the FTA-ABS results were compared with patient history (Table 1), 121 true-positive and 354 true-negative tests occurred for a reliability of 96.7%. Two false-negative FTA-ABS results were read as borderline and represented one treated case of syphilis (stage undetermined) and one case of late-latent (>2 years) syphilis. Fourteen false-positive test results represented 2 patients with other disease (unspecified) and 12 patients with no history of syphilis.

By comparing the HATTS with patient history (Table 2), the true-positive (112) and true-negative results (349) give a reliability of 93.9%. False-negative HATTS were encountered in one primary, five late-latent (>2 years), and four treated syphilis cases and in one infant with maternal antibody, for a total of 11 sera. False-positive HATTS included 1 patient with other disease (unspecified) and 18 patients with no history of syphilis. A summary of laboratory results for the nontreponemal and treponemal tests compared with patient history is presented in Table 3.

The method for comparison of syphilis history with the treponemal test results is shown in Table 4. Our analysis of the data by use of this method is presented in Table 5. The HATTS lacks sensitivity when compared with patient history of syphilis ($P < 0.01$). The specificity does not differ significantly from the FTA-ABS ($P > 0.2$), whereas the reliability differs significantly ($P < 0.01$).

The reproducibility data are presented in Table 6. The variation in the VDRL and FTA-ABS results was minimal between laboratories for all serum pools. No differences were noted between short- and long-term reproducibility for these tests. All five long-term reproducibility sera determined to be weakly reactive by the VDRL test were tested the same day. Quality assurance logs revealed that the ambient laboratory temperature was 29°C while the specimens were being tested, resulting in increased VDRL antigen sensitivity. Interlaboratory and intralaboratory variation occurred with the HATTS in serum pools C and D. Both pools were prepared to demonstrate 1+ reactivity in the HATTS. Variation of readings within pools C and D were noted between short- and long-term reproducibility studies with pool C testing nonreactive (five of five) in the short-term study and reactive (four of six) during the long-term study. Pool D tested reactive (three of five) during the short-term study, whereas it gave variable results (three of six reactive) in the long-term study.

The CDC results for the HATTS on pool C sera were ±, 1+, and 2+ for three replicates, whereas all three pool D replicates tested reactive.

DISCUSSION

The HATTS kit is nicely packaged and contains all reagents necessary to perform the test. Both 100 and 300 test kits are marketed. Laboratories may select kits corresponding most closely to their specimen work load, thereby minimizing waste due to outdated cell suspensions. Instructions are well organized and clearly written.

We evaluated the test with and without HATTS test diluent dye incorporated into the diluent during our preliminary familiarization with HATTS. The dye enables the serologist to see which wells have been diluted, thereby reducing the probability for diluting errors. We elected to perform the HATTS with the dye during all subsequent testing. We found the dye to be a useful quality assurance measure and suggest that its use should become standard for subsequently marketed kits.

Serum dilutions were prepared with a Hamilton digital diluter. With this instrument, our laboratory could perform and report 12 specimens by the HATTS in 2 h and 40 min. This number of specimens usually requires 4 h by the FTA-ABS.

Equipment required to perform the HATTS is simple and readily available in most laboratories. Although the digital diluter is not required, it reduces the time needed for preparation of the dilutions and increases the precision with which the dilutions are prepared. No incubator or fluorescence microscope with dedicated space and associated main-

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Short-term reproducibility															Long-term reproducibility											
VDRL				FTA-ABS					HATTS						VDRL			FTA-ABS				HATTS					
N	WR	1:8	1:16	N	1+	2+	3+	4+	N	±	1+	2+	3+	4+	N	WR	1:16	N	1+	2+	4+	N	±	1+	2+	3+	4+
		1	5					6						6			3				3						3
3	1					1	1	2			1	1	2		2	3					5				2	3	
5					1			4	4	1					6				3	1	2	2		3		1	
5						3	1	1		2	3				4	2			1	1	4		3	1		1	1
6				6					6						4			4				4					
4				4					4						6			6				6					

tenance costs is required since the HATTS is incubated at ambient temperature and interpreted from direct visual readings.

The staffing requirements, with regard to technical background and number of serologists required to perform the test, are equivalent for the two tests. The time required to train serologists to perform the HATTS is about 3 days compared with 5 to 10 days for the FTA-ABS. Serologists develop proficiency within 1 month with the HATTS, whereas the FTA-ABS requires 2 to 3 months of training before equivalent proficiency can be obtained. The HATTS is somewhat easier to perform than the FTA-ABS. Fewer controls are required so that set-up is straightforward and test troubleshooting is easier.

The only technical problem encountered during our evaluation was difficulty interpreting the endpoint. Handling and vibration can disrupt cell agglutination patterns, creating ambiguous reactions between negative (button) and 1+ (ring with light agglutination). Another potential source of error is delayed reading. If readings are delayed, the peripheral cells in the agglutination pattern tend to "roll" down the U-shaped well and appear as a partial button. This phenomenon is attributable to the large size of avian erythrocytes.

We noticed a slight decrease in the titer of positive control sera as a result of repeated heating. The stability of control sera is ensured by dispensing samples for daily testing.

Our reproducibility results for pools C and D illustrate the problem of endpoint interpretation by HATTS, with 12 of 22 1+ reactive sera testing nonreactive. The random distribution of results encountered with the reproducibility sera from pools C and D may have been related to single HATTS readings for each replicate tested. Normally, a questionable reaction (rough, borderline, ±, etc.) is justification for repeat testing. Since only 0.5 ml of serum was available for each replicate and the VDRL and FTA-ABS were performed before the HATTS, we were unable to repeat tests on any sera that gave a reactivity of ± in the HATTS. The lack of reproducibility for six of these sera by the HATTS results from a single subjective interpretation of borderline (±) reactivity. Difco Laboratories has produced a colored reading guide to assist serologists with interpretation of agglutination patterns since this study was completed.

The sensitivity, specificity, and reproducibility of the FTA-ABS compare favorably with previously reported results. The sensitivity of the HATTS was significantly lower than the FTA-ABS ($P < 0.01$), whereas specificity was not significantly different. This result is a function of the predominance of sera from patients who were treated for syphilis or had late-latent syphilis (82 of 123). The reliability between the FTA-ABS and HATTS was 95.1%, indicating that either test could be used as a confirmatory test for syphilis.

To summarize the advantages of the HATTS, the test is easier, faster, less expensive, and agrees favorably with the FTA-ABS. The disadvantages of the HATTS are the subjective nature of agglutination pattern interpretation and the reduced sensitivity compared with the FTA-ABS, particularly in early-primary and late-latent syphilis. The results of our reproducibility study indicate that the overall low sensitivity may be a function of reader interpretation rather than true insensitivity of the HATTS.

We found the HATTS to be a reasonable alternative to the FTA-ABS as a confirmatory treponemal test for syphilis. The FTA-ABS should be used to resolve diagnostic discrepancies which may arise in early-primary and late-latent syphilis in which the HATTS has been shown to lack sensitivity.

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