Comparative Evaluation of Three Different Treponemal Enzyme Immunoassays for Syphilis

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Background: *To reduce human errors and subjective interpretation, automation is currently a trend. However, replacing any tests with automation must first be validated.*
Objective: *Evaluate the EIA tests performance characteristics of three commercially available enzyme immunoassays; Enzygnost Syphilis (Dade Behring Ltd), Syphilis EIA 480 (Newmarket Laboratory Ltd) and ICE* Syphilis (Abbott Murex).*
Material and Method: *Three thousand and fifty- five serum samples were obtained from all workers who came for physical check ups before working abroad at the physical check-up unit of the out- patient department at Siriraj Hospital between February and August, 2001. Serum specimens known to be positive with VDRL and TPHA or FTA-ABS tests were included in the present study.*
Results: *Of all the samples, 2953 were from workers who came for physical check ups; 102 were selected from known specimens positive with the Venereal Disease Research Laboratory test (VDRL) and Treponema Pallidum Hemagglutination Assay (TPHA) or Fluorescent Treponemal Antibody ABSorption (FTA-ABS) test. A true positive result was determined when the sample was reactive either with two out of three enzyme immunoassays and TPHA or FTA-ABS, or both TPHA and FTA-ABS. A true negative result was determined when the aforementioned were absent. The sensitivity and specificity of Enzygnost Syphilis, Syphilis EIA 480 and ICE* Syphilis were 100% and 97.89%, 100% and 99.59%, and 99.1% and 99.76%, respectively. The results suggest that the specificity of Enzygnost Syphilis is the lowest among these three enzyme immunoassays; the price is also the cheapest. The decision to replace an existing test depends not only on the performance characteristics but also on other factors such as cost effectiveness, turnaround time, instrument maintenance, etc. The present study shows performance characteristics, whereas an economic evaluation is only briefly mentioned regarding a hospital's decision in making test selection.*
Conclusion: *Among the three commercial kits, the specificity of Enzygnost Syphilis was the lowest. However, the replacement of any existing test depends greatly on the purpose of the individual laboratory whereas performance characteristics will provide us with an appropriate economic evaluation.*

Keywords: *Syphilis, Treponemal Pallidum, TPHA, FTA-ABS, Sensitivity, Specificity*

J Med Assoc Thai 2006; 89 (6): 773-9
Full text. e-Journal: *http://www.medassocthai.org/journal*

A diagnosis of syphilis must be interpreted in relation to the patient's history and physical examination. Serology is the main method of diagnosis at all stages of infection although the direct detection is also used in early infection. The serological tests used most often are the nontreponemal tests (the Venereal Disease Research Laboratory test [VDRL], the Rapid Plasma Regain [RPR]) and the treponemal tests (*Treponema Pallidum* Hemagglutination Assay [TPHA] and Fluorescent Treponemal Antibody Absorption [FTA-ABS] test). Enzyme Immuno Assays (EIA) have shown some advantages in relation to the tests used for laboratory diagnosis of syphilis[1-4] since they are easy and quick to perform, objective to read and have the potential to

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be automated. The new Public Health Laboratory Service recommendations[5] extend the WHO guidelines[6], that recommend the use of a cardiolipin antigen test and TPHA for screening for syphilis, by suggesting that treponemal antigen-based EIA is an appropriate alternative to the combined VDRL/RPR and TPHA screens. The results of VDRL and TPHA are usually read subjectively, and cannot easily be automated. There is currently a trend to use automation whenever possible to reduce human error and subjective interpretation. The Clinical Laboratory Improvement Act of 1988 and the ISO 15198 International Standard, which our laboratory has been operating under, requires that whenever a new test is placed in use, it must first be validated[7,8]. Therefore, before replacing the currently used TPHA, the authors evaluated three possible replacements: Enzygnost Syphilis (Dade Behring, Marburg, Germany), Syphilis EIA 480 (Newmarket laboratory, United Kingdom) and ICE* Syphilis (Abbott murex, United Kingdom). All three tests are commercially available in Thailand. Most of the new tests have been evaluated in comparison with the standard test[9-12], e.g., VDRL and TPHA. However, no comparative evaluation of these EIA tests has been published yet. The objective of the present study was to evaluate the performance characteristics of the three EIA tests.

Material and Method

Specimens

Serum samples were obtained from all workers who came for physical check ups before working abroad at the physical check-up unit of the out- patient department at Siriraj Hospital between February and August, 2001. Serum specimens known to be positive with VDRL and TPHA or FTA-ABS tests were included in the present study. Serum samples had been stored at -20 or -70 c before the tests were performed on the same day. The exclusion criterion was inadequate specimens for all tests.

Method

All serum samples were tested with three commercial EIA kits on the same days whereas TPHA was routinely performed every day. The discrepant results between EIA and TPHA were further tested with FTA-ABS. A conclusion of true positive and negative results was as follows; true positive results when either positive in at least two out of three EIA kits and TPHA or FTA-ABS tests or positive in both TPHA and FTA-ABS tests and true negative results with the absence of positive results mentioned above.

TPHA

A commercial TPHA reagent (Newmarket Laboratory, United Kingdom) was used to detect and titrate agglutinating antibodies against *T. pallidum.* The threshold value was the titer at 1: 80. The details of the procedure and interpretation were done according to the standard technique[13].

FTA-ABS

Microscopic slides were coated with *T. pallidum*, Nichols whole antigen (Difco Laboratories, US). Specific antibodies reacting to *T. pallidum*-coated slides were detected with anti-human Ig (IgG, IgM, IgA)-fluorescent isothiocyanate conjugates (Dako, Denmark). Cross-reacting and irrelevant antibodies were first absorbed with non-pathogenic Reiters treponeme containing group treponemal antigens ((Difco Laboratories, US). The details of procedure and interpretation were done according to the standard technique[14].

The principle of Enzygnost Syphilis test is a competitive one-stage enzyme immunoassay for the in vitro determination of antibodies to *Treponema pallidum*. *Treponema pallidum*-specific antibodies (IgG and/or IgM) contained in the sample and the POD-labelled antibodies (anti- *Treponema pallidum* POD conjugate) compete for binding to the *Treponema pallidum* antigens coated onto the wells of the microtitration plate. Unbounded serum antibodies and conjugate antibodies are washed out and the enzyme activity of the bound conjugate is then determined. The enzyme component of the conjugate reacts with the Working Chromogen Solution (TMB plus hydrogen peroxide), thereby producing a blue colour change to yellow. The intensity of the resultant yellow colour is inversely proportional to the concentration of *Treponema pallidum* antibodies in the sample. This test was performed according to the recommendation of the manufacturer using the Behring ELISA processor (BEP) III that included washing, processing and reading, except specimen sampling.

The principle of the Syphilis EIA 480 test uses three recombinant antigens in a sandwich test to produce a test that is both highly specific and sensitive. The antigens will detect *Treponema pallidum* specific IgG, IgM and IgA, enabling the test to detect antibodies during all stages of infection. All reagents are supplied ready to use and colour coded. Together with the use of undiluted samples and standard volumes throughout the procedure, they are extremely

easy to use for both manual and automated assay systems. The test was performed according to the manufacturer's directions by using BEP III as well.

The principle of ICE* Syphilis method is based on the recombinant proteins TpN 15,TpN 17 and TpN 47, all labeled and carry the immunodominant epitopes from *Treponema palllidum*. The unlabelled antigens are coated on the microwells with anti-human-IgG and anti-human-IgM. Serum or plasma samples are incubated in the wells and specific antibodies against *Treponema pallidum*, if present, are captured by their related antigens on the plate. In addition, a proportion of the total IgG and IgM present in the sample is captured by the anti-human antibodies. The sample, including any unbound antibodies, is then removed by washing. In a subsequent step, a conjugate is added and captured by any specific antibody already bound to the plate. The unbound conjugate is washed away and a solution containing 3,3,5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide is added to the wells. Wells with a bound conjugate develop a purple colour that is converted to an orange colour when the reaction is stopped with sulphuric acid. The method was performed according to the manufacturer's directions by using Miniswift 91062, a fully automated machine including sampling, washing, processing and reading.

Sample size

Sample size was calculated on the assumption of prevalence of syphilis, sensitivity and specificity of the EIA test to be 1%, 95%, and 95%, respectively. Sensitivity and specificity were estimated to fall within 5% percentage points of the true value with a 95% confidence interval. The number of samples was 7,300 specimens, well over the number of test kits we had. Therefore, the authors selected some specimens positive with the VDRL and TPHA or FTA-ABS tests from samples kept during that period.

Statistical analysis

Sensitivity and specificity were determined and shown with a 95% confidence interval for each enzyme immunoassay. The McNemar test was used for a comparative evaluation between the tests.

Results

From the 3055 serum samples obtained, 2953 samples were from workers who came for physical check ups, especially syphilis testing before working abroad. The other 102 samples from specimens that were positive with VDRL and TPHA or FTA-ABS tests in our laboratory were regarded as positive specimens. Of 2953 samples from workers, 2861 were negative in all tests from the three companies and TPHA, 83 samples had discrepant results among the three commercial companies themselves or with TPHA and the other 9 samples were positive according to the criteria described earlier. Of 83 samples with discrepant results, 80 were reactive only to one test and the others positive in two tests as follows: 3 to TPHA, 6 to ICE* Syphilis, 11 to Syphilis EIA 480, 60 (32 borderline and 28 false positive) to Enzygnost Syphilis, 1 to both ICE* Syphilis and Syphilis EIA 480 and 2 to both Enzygnost Syphilis and FTA-ABS. Of these last 2 specimens, one was further performed with the Western Blot technique (MarDx Diagnostics, Trinity Biotech, Ireland) and found negative; unfortunately, the other one was not further tested due to its inadequacy as a specimen. Of the 80 samples reactive only with one EIA, 5 were randomly selected to be further performed with the Western Blot technique. Of these 5 samples, 4 reactive to Enzygnost Syphilis and 1 to ICE* Syphilis were all found negative. As a result, even though this was inadequate evidence to conclude that the true positive criteria used were correct, it supported the fact that the positive two out of the three commercial EIAs were more likely appropriate. All selective positive specimens were also positive with these three EIAs commercial kits. The results of 92 specimens composed of 9 positive specimens and 83 discrepant samples and total specimens are shown in Tables 1 and 2. The sensitivity and specificity with a 95% confidence interval were

Table 1. The samples with different results of three commercial EIAs and TPHA from 92 workers compared with the true results

EIA tests	Enzygnost Syphilis			ICE* Syphilis			Syphilis EIA 480			TPHA		
	+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-
+	8	1	0	8	0	1	9	0	0	8	0	1
True result												
-	32	30	21	7	0	76	12	0	71	3	0	80

Table 2. The samples with different results of the three commercial EIAs and TPHA from total workers compared with true results

EIA tests	Enzygnost Syphilis			ICE* Syphilis			Syphilis EIA 480			TPHA		
	+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-
+	110	1	0	110	0	1	111	0	0	110	0	1
True result												
-	32	30	2882	7	0	2937	12	0	2932	3	0	2941

Table 3. The test sensitivity and specificity of the three commercial kits and TPHA

	Sensitivity	95 % CI	Specificity	95%CI
Enzygnost Syphilis	99.1*	97.25-100	98.91*	98.55-99.27
	100.0**	-	97.89**	97.39-98.39
Syphilis EIA 480	100.0	-	99.59	99.48-99.70
ICE* Syphilis	99.1	97.25-100	99.76	99.68-99.84
TPHA	99.1	97.25-100	99.90	99.84-99.96

* When the equivocal value was considered negative
** When the equivocal value was considered positive

Table 4. The number of serum specimens in different results between the tests

	ICE* Syphilis		Syphilis EIA 480	
	+	-	+	-
Enzygnost Syphilis +	110	63	111	62
Enzygnost Syphilis -	7	2875	12	2870

	Syphilis EIA 480	
	+	-
ICE* Syphilis +	111	6
ICE* Syphilis -	12	2926

determined and are shown in Table 3. The comparison between the tests came from a number of samples with different results is shown in Table 4. A comparison between the tests suggests that Enzygnost Syphilis is statistically and significantly different from ICE* Syphilis ($p < 0.001$) and Syphilis EIA 480 ($p < 0.001$) whereas, no significant difference was demonstrated between ICE* Syphilis and Syphilis EIA ($p = 0.239$).

Discussion

All three EIAs and TPHA had similar results in very high sensitivity whereas, specificity was poorer in Enzygnost Syphilis.These results suggest that both ICE* Syphilis and Syphilis EIA 480 are useful for both screening and confirmatory tests in the worker population whereas, Enzygnost Syphilis could be used for screening but may not be appropriate as a confirmatory test. The result of ICE* Syphilis with very high sensitivity and specificity in the present study goes along with the report by Young et al[15]. But Schmidt et at[16] demonstrated that among nine different EIAs, ICE* Syphilis gave higher false positive results than Enzygnost Syphilis. This discrepant result may be because of the different confirmatory test used. In that study the FTA-ABS test, which was used as a confirmatory test, was probably insensitive enough to detect cases positive with EIAs[15]. The purpose of test applications, however, was about screening and not for confirmation; therefore, any one of these tests could be applied in our laboratory. Far more false positive and borderline results in Enzygnost Syphilis than in the others will require more time and additional budget in a laboratory, repeating other methods such as TPHA, FTA-ABS or the Western Blot to confirm the results. This will create another problem of economic concern. Enzygnost Syphilis was much cheaper than ICE* Syphilis (20 baht versus 45 baht) in similar offers, whereas Syphilis EIA 480 offered a comparable price for reagents without an automated machine. If Enzygnost Syphilis is allowed to be a service in a laboratory for routine screening on workers, the number of

confirmatory tests with TPHA or FTA-ABS (Table I: 62 cases versus 7 and 12) would be greater than the others. Nevertheless, the total cost, including reagents and labor (100 baht * [62-7 tests] versus 3055 tests * [45-20 baht]) except for overhead cost, is much cheaper than that of the others in this condition. Overhead cost was not included in this evaluation because it was the same for all the tests. If the cost included compensation for cases with false negatives that had to be sent back to Thailand, ICE* Syphilis and TPHA would be more costly than the others. Therefore, in a situation of syphilis screening for workers like these, Enzygnost Syphilis is suitable. However, this conclusion may not apply to the other population such as the blood donor group since cost determination depends on several factors such as the criteria for diagnosis differing from discarding blood, and a different prevalence etc.

The prevalence of syphilis in the worker population was very low, 0.3%. Unfortunately, since the authors did not have any patients' history records to support the diagnosis, there may have been errors in the conclusions in some cases. But in routine practice, once any specimen is positive with a screening test, a further confirmatory test will be performed and an interpretation and report will be sent to the physical check-up unit without a history check. The three commercial EIAs kits had no problem in detecting syphilis in the positive specimen group but in the workers there was a difference in both sensitivity and specificity. This shows that the cases with low antibody or no clinical symptoms must be carefully interpreted for accurate results.

The true results were determined by the UK guidelines[5] for confirmatory tests that suggest that a reactive screening result should be confirmed with a treponema antigen test of a different type from that used in screening. The FTA-ABS is not recommended as the first line confirmatory test since the specificity of the FTA-ABS is poorer than that of the other treponemal antigen screening tests[17,18] while certain newer EIAs are significantly more sensitive than the FTA-ABS[15] in detecting markers of past infection. This means that the FTA-ABS will fail to confirm a small number of genuinely reactive EIAs. The true positive specimens in the present study were those reactive to two types in the following methods: EIA, TPHA and FTA-ABS. The positive criteria for EIA were found to be positive in at least two commercial kits. Instead of two out of three EIAs as mentioned above, if positive criteria with one EIA and another test method were used, there would be little change in the sensitivity and specificity because the two serums tested by Enzygnost Syphilis would become true positive and by ICE* Syphilis and Syphilis EIA 480 would become false negative. Unfortunately, the authors performed the Western Blot on only one of these two specimens and found that it was negative. This suggested that the criteria used in the present study were appropriate since one out of two specimens was non-reactive to 4 out of 6 tests from 4 different types and the other was non-reactive to 3 out of 5 tests from 3 different types. The present study shows that if Enzygnost Syphilis is selected for routine service as a screening test, a false positive result with Enzygnost Syphilis and FTA-ABS will occur and be reported to doctors with a prevalence of 0.07% (2/2953) with 95%CI of 0.025-0.115% of all workers.

In conclusion, the present study shows that all three EIAs have comparable sensitivity but Enzygnost Syphilis has poorer specificity than the other two. Before applying each commercial kit for routine use, other factors, apart from performance characteristic, such as cost effectiveness, turnaround time, and instrument maintenance, should also be considered.

Acknowledgements

The authors wish to thank Dade Behring, Abbott Murex and Newmarket Laboratory Companies for providing the reagents and automated machine used in this study and we also wish to thank Supaporn Chaihirunkarn and Surinthorn Meepanya for their technical assistance.

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การประเมินผลเปรียบเทียบเอนไซม์อิมมูโนแอ็สเซที่จำเพาะต่อ treponema ระหว่าง 3 ชนิด เพื่อการตรวจคัดกรองโรคซิฟิลิสในกลุ่มคนงาน

รณชัย วิริยะทวีกุล, นครศักดิ์ เหลาดี, สุมาลี พจน์ประสาท, สุดารัตน์ ปิโยพีระพงศ์

ในปัจจุบันนี้การทดสอบโดยใช้เครื่องอัตโนมัติ เป็นที่นิยมเนื่องจากจะช่วยลดปัญหาความผิดพลาดที่เกิดจากการอ่านผลและการปฏิบัติงานของผู้ทำการทดสอบ แต่การจะเปลี่ยนการทดสอบใด ๆ ควรจะต้องได้รับการประเมินเสียก่อน น้ำยาทดสอบสำหรับการตรวจหาซิฟิลิสโดยเครื่องอัตโนมัติที่มีจำหน่ายในประเทศไทย 3 ชนิด คือ Enzygnost Syphilis (Dade Behring Ltd) Syphilis EIA 480 (Newmarket Laboratory Ltd) และ ICE Syphilis (Abbott Murex). ได้ถูกนำมาประเมินคุณสมบัติของการทดสอบโดยใช้ตัวอย่างน้ำเหลือง 3,055 ตัวอย่าง ซึ่ง 2,953 ตัวอย่าง มาจากกลุ่มคนงานที่มาตรวจสุขภาพและอีก 102 ตัวอย่าง มาจากตัวอย่างน้ำเหลืองที่เคยให้ผลบวกต่อ Venereal Disease Research Laboratory test (VDRL) และ Treponema pallidum hemagglutination assay (TPHA) หรือ fluorescent treponemal antibody absorption (FTA-ABS) ผลบวกที่แท้จริง คือตัวอย่างน้ำเหลืองที่ให้ผลบวก จาก 2 ใน 3 ของน้ำยาทดสอบที่ถูกประเมินและ TPHA หรือ FTA-ABS หรือตัวอย่างน้ำเหลืองที่ให้ผลบวกต่อ TPHA และ FTA-ABS ขณะที่ผลลบที่แท้จริงคือ ตัวอย่างน้ำเหลืองที่ไม่ได้ผลดังกล่าวข้างต้น ผลการวิจัยพบว่าความไว และความจำเพาะของ Eznygnost Syphilis, Syphilis EIA 480, and ICE* Syphilis เป็นดังนี้ คือ 100% และ 97.89%, 100% และ 99.59% และ 99.1% และ 99.76 % ตามลำดับ ซึ่งจะเห็นว่าค่าความจำเพาะของ Enzygnost syphilis ต่ำสุด ขณะที่ราคาถูกที่สุดด้วยเช่นกัน การจะตัดสินใจเลือกการทดสอบใดว่าเหมาะสม นอกจากจะขึ้นอยู่กับคุณสมบัติของการทดสอบแล้ว ยังควรพิจารณาปัจจัยอื่น ๆ ด้วย เช่น ความคุ้มค่า ช่วงเวลาที่ใช้ในการทดสอบ การดูแลรักษาเครื่อง ในการศึกษานี้ได้แสดงถึงผลของคุณสมบัติการทดสอบ ส่วนการประเมินด้านความคุ้มค่ามีการกล่าวถึงบ้างเล็กน้อยในมุมมองการตัดสินใจของโรงพยาบาล โดยสรุป ในระหว่าง 3 บริษัท ที่ทำการศึกษา ค่าความจำเพาะของ Enzygnost syphilis ต่ำสุด แต่อย่างไรก็ตาม การเปลี่ยนการทดสอบจะขึ้นอยู่กับวัตถุประสงค์ของห้องปฏิบัติการนั้น ๆ เป็นสำคัญ ซึ่งคุณสมบัติการทดสอบจะช่วยให้เป็นข้อมูลในการนำไปการประเมินในเชิงความคุ้มค่าได้อย่างเหมาะสม*