Development of Elisa for Rapid Detection of Anti - Dengue Antibody

Amin Soebandrio W.K., dr, Ph.D.

Abstrak

Demam Berdarah Dengue (DBD) merupakan masalah kesehatan masyarakat yang cukup serius dengan angka kesakitan dan angka kematian yang cukup tinggi di Indonesia dan banyak negara lainnya. Diagnosis dini dan pengobatan segera merupakan kunci keberhasilan pengobatan. Cara-cara diagnosis yang ada saat ini membutuhkan prosedur yang rumit dan memakan waktu, sehingga tidak dapat memberi kepastian diagnosis penyakit ini pada stadium awal. Dalam makalah ini akan dilaporkan suatu usaha untuk mengembangkan suatu prosedur diagnostik cepat yang berdasarkan deteksi IgM anti dengue melalui cara ELISA, dengan penekanan pada pengembangan cara pelapisan plat ELISA dengan virus dengue sebagai antigen. Dari 151 serum penderita tersangka DBD yang ditapis dengan uji hambatan hemaglutinasi selama masa 10 bulan, 37 persen dipastikan berasal dari penderita infeksi dengue, dan diantara mereka 12 memenuhi persyaratan untuk proses berikutnya. Hasil-hasil menunjukkan bahwa virus dengue tip 2 (DV-2) lebih baik dari tip 1 (DV-1) sebagai antigen pelapis, sementara antigen yang dilapiskan pada suhu 37° C mampu mengikat antibodi sesuai dengan dosis yang diberikan, lebih baik daripada yang dilapiskan pada 4°C. Pengikutsertaan kompleks Avidin-Biotin dalam proses pelapisan secara bermakna meningkatkan kemampuan campuran antigen DV-1 dan DV-2 dalam mengikat antibodi anti-dengue. Pembandingan titer HI dengan ELISA menunjukkan bahwa antibodi ELISA berbeda dari antibodi HI. Hasil-hasil yang diperoleh menunjukkan bahwa prototip sistim ELISA untuk mendeteksi IgM anti-dengue telah berhasil dikembangkan, walaupun untuk penerapan di lapangan secara lebih luas masih membutuhkan penelaahan lebih lanjut.

Abstract

Dengue Hemorrhagic Fever (DHF) is a major public health problem with high morbidity and motality rate in Indonesia and many other countries. Early diagnosis and prompt treatment are the key to successful therapy. Diagnostic methods available widely require laborious and time consuming procedures, and can not provide confirmation of the desease in the early stage. In this paper will be reported an attempt to develop a rapid diagnostic procedure based on the detection of anti-dengue IgM by the ELISA method, with emphasis on the development of a coating method of the ELISA plate using dengue virus as the antigen. From 151 sera of dengue suspected patients screened by Hemagglutination Inhibition (HI) test in a period of 10 months, 37 percent were confirmed to be from dengue infected individuals and among them 12 were eligible for further procedures. Results showed that dengue virus type 2 (DV- 2) is superior to those of type 1 (DV-1) as the coating antigen, while antigen coated at 37°C showed better ability to bind antibody in a dose dependent manner compared to those done at 4°C. The involvement of the Avidin-Biotin complex in the coating process significantly increased the ability of DV-1 and DV-2 mixtures to bind antidengue antibody. Comparison of HI and ELISA titers revealed that the ELISA antibody is different from HI antibody. These results showed that the prototype of the ELISA system for detection of antidengue IgM has been successfully developed, although for wider and field applications further studies still need to be done carefully.

Keywords : *Enzyme Linked Immuno-Sorbent Assay; Hemagglutination Inhibition; Dengue Virus.*

INTRODUCTION

Dengue fever is a self-limiting disease with a variety of symptoms such as fever, headache, muscle and joint pains, lymphnode enlargement and leucopenia caused by the dengue virus, a microorganism from the Flavovirus family transmitted by the Aedes aegypti mosquito.[1,2,3] Wuryadi[4] reported that in Indonesia the dominant virus types are type 2 and 3, the latter being related to the Dengue Hemorrhagic Fever (DHF).

Dengue Hemorrhagic Fever (DHF) is a severe and life-threatening form the Dengue Fever, the pathogenesis and pathophysiology of which have not been understood completely. Treatment consists only of symptom control and the prevention of complications. Mortality of DHF patients reaches 50 percent

Department of Microbiology, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia

without immediate treatment but decreases to 5 percent with immediate and adequate therapy based on accurate diagnosis and management. A rapid and accurate method of diagnosis is needed for specific identification, before clinical symptoms are distinct.

MATERIALS AND METHODS

ELISA

The Enzyme-Linked Immunosorbent Assay used in this investigation followed the recognized method used by many institutions. [7]

Coating of ELISA wells

a. Testing the effect of temperature on coating efficiency.
Dengue Virus type 1 and 2 stock with a titer of HA 1024 was diluted a hundred-fold in PBS-Azide containing 0.1 percent BSA,distributed into wells in amounts of 200 µl per well followed by an overnight incubation at 37°C or 4°C. The virus-containing fluid was then discarded, and the wells subsequently blocked with 1 percent BSA in PBS-Azide for 60 minutes at 37°C. The wells were then washed four times with PBS- Azide prior to the implementation of further steps in the ELISA procedure. Efficiency was evaluated by the ability to bind and differentiate various concentrations of anti dengue IgG. During the preliminary study, anti dengue IgM was not found to bind sufficiently to the dengue virus coated at low temperature and was therefore not included in this study.

b. Testing the effect of Avidin-Biotin binding on coating efficiency.
Each well was precoated with 200 µl Avidin (Boehringer Mannheim) at a concentration of 10 ug/ml in PBS-Azide.
Blocking was done with 1 percent BSA in PBS-Azide for 60 minutes at 37°C, 60 µg/ml Biotin conjungated dengue viral antigen in PBS Ag was added, followed by an overnight incubation at 37°C. The wells were then washed 4 times with PBS-Azide before further steps in the ELISA procedure were implemented.
Efficiency was evaluated by measuring the capacity of binding and differetiation of various concentrations of anti dengue IgG and IgM. Both immunoglobulins were used in this experiment because in the preliminary investigation both were found to exhibit a tendency to increase the efficiency in the presence of Avidin- Biotin binding.

In the two above mentioned coating methods, the coated plates were kept in a humid container at 4°C until further use.

The dengue viral antigen

The antigen for the HI (Hemagglutination inhibition) test to be used was of the type 2 variety derived from mouse brain and obtained from Biofarma. For ELISA, type 1 and 2 virus were purified by the author from infected mouse brain.

Propagation and isolation of dengue virus

The virus was propagated by injecting a virus stock diluted in sterile PBS into the brains of baby mice in doses of 0.1 to 0.2 ml. After three to seven days, the mice were killed when neurological symptoms such as loss of balance or sickness developed, and the brain subsequently extracted. If immediate processing was not possible,the mice were kept at - 70°C. Twenty mice were used for each viral type.

To obtain the virus, the mice brain tissues were processed with a standard method.[8,9] Several modifications were employed : mouse brain tissue suspended in cold 0.02M Tris HCl was centrifuged at 8500 g for 15 minutes at 4°C, one ml of supernatant was mixed with 5 mg Protamin Sulphate (Sigma), and was left to stand at 4°C for 30 minutes, occasionally shaking the mixture; then centrifuged at 3000 g for 15 minutes at 4°C. To 1 ml of supernatant 3.8 g heparin was added to inactivate the remaining protamin sulphate.

The virus in the supernatant was then extracted by centrifugation at 3000 g for 15 minutes at 4°C. The titer of the virus was determined by hemagglutination.[5]

Conjugation of viral antigen with biotin

Biotin was obtained as the commercial preparation N-Hydroxy Succinimid Biotin (NSB - Boehringer Mannheim). Conjugation was performed according to the standard method[10,11,12] : 0.1 ml of a 1 mg/ml Biotin solution in DMF (Dimethyl Formamide) was slowly added dropswise to 0.1 ml of a viral antigen solution with a titer of 1024 , then left to react for 2 hours at room temperature. After dilution to 1 ml with PBS the solution was extensively dialyzed changing the PBS-azide 3 to 4 times at room temperature. The concentration of the antigen biotin conjugate was determined spectrophotometrically at a wavelength of 280 nm.

Patient's Sera

The sera of DHF patients were obtained from hospitals or practitioners sent to the microbiology laboratory of the University of Indonesia Faculty of Medicine during the 10 months research period.

Because of the high variety of patients and the difficulties in monitoring the actual clinical course of the disease, the first (I) serum specimen was considered as serum in an acute state (acute serum) whereas the second (II) serum as convalescent serum. Sera which met the WHO criteria[6] for primary or secondary viral infection were tested.

Hemagglutination (HA) and Hemagglutination Inhibition (HI)tests. The Hemagglutination test for the titration of the virus and the hemagglutination inhibition test on all sera of suspected DHF patients were done according to standard procedures[5,13], as follows :the antigen used was a crude preparation of inoculated mouse brain. Titration of antigen was done by hemagglutination at various pH, performed by mixing various concentrations of antigen at different pH with equal amounts of 0.4 percent goose erythrocyte suspension into round bottomed wells. The titer of the antigen was determined from the highest antigen dilution which could still show agglutination of the goose erythrocytes. A patient's serum or plasma was first extracted with kaolin to eliminate nonspecific and natural agglutinins which could potentially disturb the hemagglutination process. Antigen with a titer of 4-8 units at optimum pH was then mixed with an equal amount of tested serum and after an overnight incubation at 4°C added with an 0.4 percent goose erythrocyte suspension in the same amounts as above. The HI titer was determined from the highest dilution which could still show near or complete inhibition. For each test a repeat titration of antigen, control serum, and antibody using a standard serum was performed. Then HI test was performed on serum I and II (acute and convalescence) run concurrently for comparasion of the differences. Interpretation was done in accordance with the WHO standard of 1986[6] as seen in Table 1.

RESULTS AND DISCUSSION

Screening of sera of suspected patients

During the 10 months of this study 151 blood specimens were obtained from patients suspected to have dengue fever. After screening with the hemagglutination inhibition test, only 37 percent of the patients were confirmed to have dengue fever, primary or secondary infection. As seen in Table 2, no conclusion could be drawn from approximately 62 percent of the sera due to various reasons, i.e. no increase in titers of sera I and II and a titer of less then 1280 for serum II.

Table 1. Interpretation of the hemagglutination inhibition test for dengue fever

Interval	Increment	Titer Serum II	Conclusion
>/= 7 days	>/= 4 x	</= 1 : 1280	Primary infection
Variable	>/= 4 x	>/= 1 : 2560	Secondary infection
< 7 days	>/= 4 x	</= 1 : 1280	Primary or secondary infection
Variable	none	>/= 1 : 2560	Probable secondary infection
>/= 7 days	none	</= 1 : 1280	Not dengue
< 7 days	none	</= 1 : 1280	No conclusion
only one serum		</= 1 : 1280	No conclusion

WHO 1986.[6]

In several cases serum II was taken too early. In such situations diagnostic confirmation could still have been sought with a "third" serum taken five days after the second serum. In still other cases the second serum was not received.

Table 2. Results of the hemagglutination inhibition test on 151 sera of suspected patients.

Interpretation	Total	%
Dengue infection (primary or secondary)	56	37.1
Probable dengue	2	1.3
No Conclusion	93	61.1

note : interpretation based on WHO criteria 1986.[6]

Of the 56 (37 percent) confirmed sera, only 12 could be used for all the tests, the others being inadequate in amount.

The sera were from patients number : 5, 13, 22, 26, 29, 30, 32, 34, 39, 98, 101, and 104. The inadequate amount of blood /sera specimens was mostly due to the fact that the majority of patients were children, thus it was more difficult to obtain the needed amount of venous blood.

Difference between dengue virus type 1 and 2 in their ability to bind anti-dengue antibody

As discussed earlier, the predominant types of dengue virus in Indonesia are type 2 and 3, followed by type 1 and 4.

From various reports and investigations on the pathogenesis, it was found that there exists a very strong cross reactivity among types 2,3, and 4. Based on these data, dengue virus type 2 has been chosen for the ELISA system presently being developed in our laboratory to detect the anti-dengue antibody. For confirmation, the binding ability of type 2 dengue viral antigen to anti-dengue antibody was compared to that of type 1.

Table 3. Ability of DV-1 and DV-2 antigens to bind anti-dengue antibody**

Test serum*	IgG		IgM	
	DV-1	DV-2	DV-1	DV-2
I-13	0.272	0.720	0.214	0.269
I-22	0.259	0.496	0.216	0.390
I-26	0.270	0.536	0.244	0.290
I-30	0.330	0.595	0.226	0.376
	$p < 0.01$		$p < 0.05$	

notes : DV-1 : dengue virus type 1
DV-2 : dengue virus type 2
* : batch number of tested acute serum
** : results expressed as OD492

Table 3 shows that viral antigen type 2 has a greater ability to bind IgG and IgM, expressed as the higher OD 492 readings. Using the limited sample available,statistical analysis revealed a sinificant difference. The superiority of type 2 virus in this investigation was caused by the greater affinity of anti dengue antibody to DV-2 or other types of dengue virus (cross reaction).Another reason might be that the sample population was predominantly of the type 2 dengue virus. This should be proven by using a larger sample population and a test, more specific for each type such as the neutralization test. This test was, however, not done in this investigation due to its complexity and high cost. In our study we only used the first or acute sera with the aim of obtaining high OD 492 readings for both IgG and IgM, avoiding the low general IgM readings for the second sera (convalescent) found in the preliminary investigation.

Effect of the Avidin-Biotin complex on the efficiency of the dengue viral antigen binding to anti dengue antibody.

Protein molecules are characterized by their strong affinity to plastic surfaces, probably caused by the interaction between the nonpolar protein structure and the plastic matrix, forming the basic principle for coating the ELISA plate with protein molecules.[7] However, it is also recognized that in one protein molecule more than one non polar part can be found, each having equal chance of adhering to a plastic surface.

As a results, the protein molecules will adhere to a plastic surface at random with a heterogenous orientation, depending on the coating process. This phenomenon occurs also in protein antigen molecules with more than one epitope. As a certain antibody will only recognize a specific epitope, it is undertandable that an antigen coating having a heterogenous orientation will result in a low efficiency of binding with its antibody, aside from the inconsistency of the reaction itself at different times.

Efforts were made to overcome the problem by endeavoring a homogenous orientation.

Biotin, known also as the coenzyme R or vitamin H has unique structure and caracteristics and with a simple technique, it is easily bound to a protein molecule.

This study used Biotin having a spacer arm so that its binding with a protein molucule will not distrub the other reactions i.e. between Biotin and other subtances or between the protein molecules. Avidin is a glycoprotein tetramer isolated from egg white or a bacteria species (for streptavidin) with a high and specific affinity for biotin, higher than antigen - antibody binding. Each avidin molecule can bind 4 molecules of biotin irreversibly except in extreme conditions[16]. Like other proteins, avidin adheres to plastic surfaces. If a biotin conjugated antigen protein solution is incubated in plastic wells previously coated with avidin, the antigen protein will be strongly bound with a homogenous orientation, so that subsequent exposure to an antibody will also result in a homogenous reaction.

Another advantage of utilizing avidin - biotin is the stability of the protein antigen bond to a plastic matrix resulting in resistance to storage or physical challenge such as washing etc.

In this investigation a similar procedur was performed on viral antigen with directly coated antigen. The results were as expected. As seen in table 4 ELISA plates coated with a combination of virus type 1 and 2 and the assistance of avidin-biotin complex revealed a significantly higher efficiency ($p=0.01$) in binding antidengue IgG.

With IgM, an incompatible result was found in one of the test sera (I-30), where the avidin-biotin complex seemed to have decreased the efficiency of IgM in binding to the dengue virus. This apparently

was the cause for the statistically non significant difference between the avidin-biotin treatment group and the untreated group although individual observation showed that there is a tendency towards a difference. At present no conclusions can be made as to whether this incompability was due to technical reasons or to the pentameric structure of IgM itself.

Table 4. Effect of the Avidin-Biotin bond on the ability of viral type 1 and 2 viral antigen mixture to bind anti dengue antibody**

Test serum*	IgG		IgM	
	(A-B) -	(A-B) +	(A-B) -	(A-B) +
I - 22	0.355	0.526	0.280	0.434
I - 30	0.453	0.547	0.353	0.297
I - 34	0.480	0.525	0.345	0.379
I - 101	0.454	0.539	0.408	0.422
I - 104	0.383	0.553	0.362	0.467
	p = 0.01		p > 0.2	

(A-B)- = antigen directly coated without avidin-biotin
(A-B)+ = antigen conjugated with biotin, then bound to avidin coated ELISA plate. For further details, see materials and methods
* : I indicates acute serum tested
** : results expressed as OD492

In this test the combination of dengue type 1 and 2 was used as there are plans for future research in which the combination will be used in large amounts as ELISA plate coating antigen with the assistance of avidin-biotin.

It seems that the test will have to be backed up by data from each viral type and a larger population of test sera, to obtain more conclusive data on the advantages of the avidin-biotin complexially with regard to the binding efficiency of IgM antidengue, as the ELISA system is being developed for the detection of IgM kinetics.

Effetcs of coating temperatur on anti-dengue antibody binding

The velocity and the affinity of the protein molecule to adhere to a plastic surface depend on many factors such as the distribution coefficient of the molecule, the ratio between the area of the surface to be coated and the volume of the coating solution, the concentration of the coating agent, the temperature and the length of time of the coating process[7]. By using the same solution with the same concentration and volume, the only remaining variables are temperature and reaction time. If the reaction time can also be controlled, then only the temperature will be left for consideration. The amount of antibodies bound will be directly proportional to the amount of antigens present on the plastic matrix, so that the coating efficiency can be assessed by quantitating the antibodies bound. To determine the effect of temperature, coating with dengue viral antingen was performed at two different temperatures, i,e, low (4°C) and a relatively higher temperature (37°C).

Table 5, shows that by coating at 37°C more antigens were bound to the plastic matrix, shown as the amount of bound antibodies. A significant difference was found for both type 2 ($p < 0.02$)and type 1 ($p < 0.01$). Since the ELISA system is to be used to measure anti-dengue antibody, at least semiquantitatively, the efficiency of the coating procedure needs further evaluation, using different amounts of antibodies.

The sensitivity of this procedure was tested using antibodies diluted to 1/20 and 1/40.

Table 5. Effects of coating temperature on anti dengue antibody binding * *

Test Sera * (IgM)	DV-1		DV-2	
	37°C	4°C	37°C	4°C
II - 30	0.338	0.257	0.466	0.329
II - 39	0.347	0.282	0.380	0.312
II - 13	0.430	0.315	0.488	0.324
II - 32	0.420	0.288	0.433	0.221
	P < 0.01		P < 0.02	

DV-1 : dengue virus type 1
DV-2 : dengue virus type 2
* : batch number of tested convalescent serum
** : results expressed as OD 492

Table 6. Effects of DV-2 coating temperature on binding at various concentrations of anti-dengue antibody. * *

Test Sera * (IgG)	37°C		4°C	
	1/20	1/40	1/20	1/40
II - 30	0.416	0.297	0.329	0.288
II - 39	0.381	0.282	0.312	0.278
II - 13	0.488	0.305	0.324	0.281
II - 32	0.433	0.280	0.291	0.260
	P < 0.005		P < 0.005	

DV-2 : Dengue virus type 2
* : batch number of tested convalescent serm
** : results exprssed as OD 492
For futher details, see materials and methods.

For the ELISA plate coated with dengue virus type 2, dilution 1/20 and1/40 could be differentiated significantly ($p < 0.005$ for coating at 4°C and 37°C) as seen in table 6. For virus type 1, differentiation between 1/20 and 1/40 was possible only when antigen was coated at 37°C shown in Table 7.

Table 7. Effects of DV-1 coating temperature on binding at various concentrations of anti-dengue antibody * *

Test Sera * (IgG)	37°C		4°C	
	1/20	1/40	1/20	1/40
II - 30	0.338	0.291	0.354	0.264
II - 39	0.347	0.270	0.282	0.259
II - 13	0.430	0.294	0.315	0.280
II - 32	0.415	0.280	0.288	0.267
	$p < 0.05$	$p > 0.01$		

DV-1 : dengue virus type 1
* : batch number of tested convalescent serum
** : results expressed OD492
For futher details, see materials and method.

In the aforementioned tests the dengue virus type 2 was shown to have equal binding capability at both temperatures.

Coating at 37°C was chosen in subsequent tests due to superior results for both types of viruses. Incubation at room temperature (24°C - 27°C) is presently under testing in our laboratory.

Correlation between HI antibody titer and ELISA

After invading the human body, the dengue virus stimulates the production of antibodies that inhibit hemagglutination (HI antibodies), antibodies that neutralize the virus (Nt antibodies) and antibodies that fix the complement[1].

No information is yet available on which antibody is involved in the ELISA system. Comparative studies were done between HI antibody kinetics and anti-dengue IgG and IgM detected by ELISA in the acute phase. For example, serum 5 which did not meet the WHO criteria for dengue infection was found to have a high level of ELISA antibodies, almost as high as serum 98 which met the criteria of a four-fold titer increase, even with the convalescence serum titer remaining low.

This applied also to serum 30 which had a high HI titer but no subsequent increase. On the other hand, sera which were proven to have significant differences between the acute and convalescent phases were not acommpanied by the same tendencies in detectable antibody (IgG and IgM) levels. Sera 13 and 29 for example, had significantly high levels of IgG but not IgM. Several other sera such as number 39 and 104 showed high ELISA antibody titers with four-fold increases of HI titers and a significant high convalescent serum titer.

Table 8. Correlation between HI antibody titer and ELISA antibody titer

Test Sera	HI		ELISA	
	Acute	Con	IgG	IgM
5	80	80	0.600	0.333
13	80	1260	0.721	0.269
29	20	2560	0.452	0.267
30	2560	2560	0.595	0.376
39	160	2560	0.840	0.486
98	20	160	0.614	0.372
101	160	1280	0.674	0.544
104	160	1280	0.802	0.684

Coating with DV-2 at 37°C without avidin-biotin and only acute sera tested. Result expressed as OD 492.

From these observations several conclusions were drawn :firstly, the ELISA antibody is not necessarily the same as HI antibody : secondly, the ELISA antibody might be a combination of antibodies present; thirdly, since the HI test is still the accepted routine test and recommended by the WHO, the ELISA antibody titer will have to be determined against the cut off value of the HI test to confirm dengue infection.

DISCUSSION

Sera from 151 suspected DHF patients were obtained during a period of ten months. This number is small and not in agreement with the incidence of DHF in Jakarta. For future studies case finding in the field and cooperation with clinicians should be intensified. In the ELISA test no control sera, positive or negative, were used, because as far as we know there has not been any investigation into the levels of anti-dengue IgG nor IgM antibodies in Indonesia, so no information is yet available on the antibody status of Indonesians, proper for the determination of the cut off value.

This has posed difficulties in standardizing the antibodies. As an alternative, a buffer solution was used as the negative control to replace a test serum. Several possibilities were considered to be used as control serum, among the most contemplated were sera taken from the healthly population. It is hoped that the ELISA method being developed will help to determine

the standard levels for anti-dengue IgG and IgM in Indonesia.

Several investigators have attempted to apply ELISA to detect IgM anti-dengue antibody using cells infected with dengue virus as the antigen.[17,18] The advantage is that the virus does not have to be purified prior to use and up to a certain limit the antigen is easier to coat on the ELISA plate as several types of cells adhere to plastic matrix.

The disadvantages are the difficulty to determine the numbers of antigens in each cell,and the greater possibility of non specific reactions with the cell components or the medium. These reasons have been the basis of the choice of the protamine sulphate purified virus[8,9] as the antigen coating the ELISA plate.

The various tests performed in this study have shown that in a serum specimen the level of detectable anti-dengue IgM is always lower than that of IgG. This may be simply because IgM anti-dengue is found in lower concentrations in relation to the clinical course of the disease. However IgM and IgG anti-dengue antibody both present in the serum compete for binding to the dengue viral antigen in the same ELISA well.

The greater binding affinity of IgG to its antigen compaired to that of IgM explains why IgM is detected in smaller amounts than IgG. To overcome this problem, the ELISA procedure could be modified with the "IgM capture" system[19] where only IgM react with antigen without competition from IgG. This technique is superior in detecting IgM, but has its consequences. Another method is the detection of IgM both in the free and bound (to virus) states, a technique based on the binding of IgM to the virus early in the disease state.[20]

The sensitivity of the ELISA method needs further study, the optimum serum dilution, the smallest amount of detectable anti-dengue antibody and the earliest detectable phase of the disease is still to be evaluated. Sensitivity will have to be acommpanied by acceptable specificity to avoid the possibility of cross and non-specific reactions. The specificity of results in this study still has to be assessed with the neutralization test and a field trial on a larger scale will have to be implemented before this method can be applied for practical use.

In this study, incubation of patients sera was done overnight; in another study (unpublished), the author found that an incubation time of two hours, for test sera and antibody-enzyme conjugates, had results comparable to that found in this investigation, which is very significant as the system was developed to give results in the shortest time possible. When this system has been fully developed, test results are expected to be available to the clinician within two hours.

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