Original Article

Comparison of the combination of recomLine and ELISA with Real-Time Polymerase Chain Reaction In The Final Diagnosis Of Toxoplasmosis

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Summary:

Background: The diagnosis of Toxoplasma gondii infection in human can be determined by variable immunological and molecular methods. The methodology for antibodies detection is well established allowing the identification of serological profile correlated with the stage of the infection. The recomLine Toxoplasma is designed as a confirmation test for serologic diagnosis. The line assay technique facilitates antibodies detection and identification in a single test. While real-time PCR has recently been introduce for the diagnosis of toxoplasmosis in a short time.

Patients and Methods: Twenty women who had a history of abortion and infected with acute toxoplasmosis were firstly diagnosed by ELISA IgM Toxoplasma antibodies. Then the diagnosis was confirmed by recomLine Toxoplasma IgM .Finally real time-PCR was done to each women.

Results: The results showed the utility of combination of ELISA and recomLine in the diagnosis of toxoplasmosis than using each test alone in the diagnosis, or using RT-PCR. The sensitivity of the parallel combination of ELISA and recomLine assays was 80%, while the sensitivity of RT-PCR was only 40%.

Conclusion: Detection of IgM- Toxoplasma antibodies by ELISA lead to false positive results, a confirmatory test like recomLine assay which is satisfactory test that offer the same advantage of immunoblotting in the diagnosis of acute toxoplasmosis. A new tests system that include the combination of ELISA and recomLine assays, had high sensitivity compared to the sensitivity of RT-PCR, and can be used in the diagnosis of acute toxoplasmosis.

Keywords: recomLine assay, Real-Time-PCR, acute toxoplasmosis .

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Introduction:

Toxoplasmosis is caused by the parasite Toxoplasma gondii , the course of an infection of an immunocompetent individual with T.gondii is usually asymptomatic , with rare development of non specific flu like symptoms, serologic evidence indicates that human infections are common in many parts of the world. Although adult-acquired toxoplasmosis is usually mild to asymptomatic, the disease can be severe in the immunocompromised, leading to encephalitis (1). The initial infection results in life long persistence of the parasite and confers life long immunity to further toxoplasmosis infections (2). Congenital toxoplasmosis may cause serious damage to the fetus. The probability of transmission increase, although the severity of the clinical manifestations decreases, as the pregnancy progresses. Drug therapy can be initiated if an initial infection is confirmed in the course of pregnancy since the course of toxoplasmosis is generally asymptomatic, the diagnostic procedure is generally based on serological methods (3). The detection of IgM antibodies is not sufficient for the diagnosis of acute toxoplasmosis. Therefore, using of recombinant antigens in recomLine assay, which are taken both from the tachyzoites dominating the productive infection phase and from the bradyzoites or from both forms which facilitate confirmation of infection status (2,4,5,6). Real-time PCR has been recently used for the diagnosis of toxoplasmosis, combine the steps of the amplification , are product detection in a single phase thereby reduce the time from 24 to 48 hours to less than 4 hours (7, 8, 9)

Subjects: The blood sample from twenty women with history of single or recurrent abortions were analyzed for detection IgM of T.gondii by ELISA and recomLine assay for detection IgM of T.gondii , real-time PCR was done for detection DNA of T.gondii. The samples were collected during the period between May 2008 and November 2009. The range of the age was 15-40 years. Kits used in the present study, ELISA kit for detection IgM-Toxoplasma antibodies. BioCheck,Inc. / USA, (recomLine Toxoplasma IgM kit) provided by Mikrogen Company/Germany, and Real-time PCR kit provided by Sacace /Italy, using fluorescent reporter dye probes. The sequence of the primers targeting 529bp element were 5-CACAGAAGGGACAGAAGT and 5-TCGCCTTCATCTACAGTC .

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Methods: The recomLine test kit Toxoplasma is a qualitative in vitro test for the detection of IgM antibodies. in human serum against protein antigens of T.gondii is done. Due to the use of various
recombinant antigens, the assay gives information on the specific antibodies at a glance which are allocated to the various stages of Toxoplasma status, the ROP-1, GRA-7 and GRA-8 the most antigens associated with IgM-Toxoplasma antibodies (2,3) .
Real-time PCR is a quantitative test for detection of DNA of T.gondii in the biological samples, amplified using real-time amplification with fluorescent reported dye probes specific for T.gondii and Internal Control(IC).The target used was 529bp which occur in 200300- copies (10). Final diagnosis of toxoplasmosis was depend on the positive results of recomLine and ELISA and /or real-time PCR assays .
ELISA was used for detection Toxoplasma-IgM for human serum , purified Toxoplasmna antigen is coated on the surface of microwell, diluted patients sera were added to the well and the Toxoplasma IgM-specific antibodies bind to the antigen(11).
Statistical analysis: Statistical analyses were computer assisted using SPSS . The performance characteristics (validity) of a test or criteria, include among others: sensitivity, specificity, positive predictive value and negative predictive value. Also, ROC analysis curve which give the value of Are Under Curve (AUC) to select the best cut off value that illustrated the validity of test used to diagnose the infection.

Results:
To asses different techniques applied for diagnosis of toxoplasmosis, women with single or recurrent abortion were included in the present study to determine the performance indices of the available tests named recomLine, ELISA, Real-Time PCR. Table (1) shows the Receiver Operating Characteristic (ROC) analysis of serum IgM Toxoplasma antibodies detected by ELISA, the value of Area Under Curve (AUC) was 0.685, which was not statistically significant, (P=0.16).

Table (1): The ROC area for selected parameters when used to diagnose toxoplasmosis

	Area	Probability
IgM Toxoplasma Abs by ELISA	0.685	0.16 (NS)

Table (2) shows ROC analysis to select the typical cut off value, which was 0.95. The sensitivity was 90%, specificity was 60%, with accuracy 75% .The diagnosis of toxoplasmosis with 60% confidence in a clinical situation where the disease is of high probability at (40% pretest probability). The confidence level in positive tests much low 20% at (10% pretest probability).A negative test in the same clinical situation 10% pretest probability would exclude the diagnosis with 98.2%.

Table (2): The validity parameters of serum IgM –Toxoplasma antibodies (Acute reaction) by ELISA when used as a test to diagnose toxoplasmosis

				PPV at pretest probability =		NPV at pretest probability = 10%
Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	10%	40%	
IgM Toxoplasma antibody						
0.790	100.0	81.8	86.7	37.9	78.6	100.0
0.950	90	60	75	20	60	98.2
1.034	58.3	90.9	82.2	41.6	81.0	95.2

PPV: Positive Predictive Value, NPV: Negative Predictive Value.
The results shown in Table (3) indicate that the cut off value of positive IgM –Toxoplasma antibodies detected by ELISA was 1.022 was nearly compared to the value of the typical cut off value 0.95,this cut off value was used to differentiate positive results from the negative results.

Table (3): The association between IgM Toxoplasma antibodies test (acute reaction) by ELISA and final diagnosis of toxoplasmosis

IgM Toxoplasma Abs detected by ELISA	Final diagnosis of toxoplasmosis		
	Negative	Positive	Total
Negative	6	1	7
Positive	4	9	13
Total	10	10	20

Sensitivity=90.0%(54-99%);Specificity=60.0%(27-88%);accuracy=75.0%(51-90%); PPV at 10% pretest probability =20.0%; PPV at 40% pretest probability =60.0%; NPV at 10% pretest probability =98.2% The sensitivity, specificity, and accuracy of IgM –Toxoplasma antibodies detected by ELISA were similar to that in Table (2) with typical cut off value determined by ROC analysis , also with the same positive test at (40% pretest probability) and at (10% pretest probability), and in the negative test which was 98.2% confidence to exclude the presence of the disease.
The results in Table (4) show the validity of recomLine Toxoplasma-antibodies assay that was used to diagnosis toxoplasmosis.

Table (4): The validity parameters for positive recomLine test when used as a test to diagnose toxoplasmosis.

	Final diagnosis of toxoplasmosis		
recomLine test	Negative	Positive	Total
Negative	8	1	9
Positive	2	9	11
Total	10	10	20

Sensitivity=90.0%(54-99%);Specificity=80.0%(44-96%);accuracy=85.0%(61-96%); PPV at 10% pretest probability =33.3%; PPV at 40% pretest probability =75.0%; NPV at 10% pretest probability =98.6%.

The sensitivity of recomLine assay was 90.0 %, the specificity was 80.0%, with accuracy 85.0 % .The positive test can establish the diagnosis of toxoplasmosis with 75.0 % confidence in a clinical situation when the disease is of high probability (40 % pretest probability) .The confidence level in positive test is much lower (33.3 %) in a clinical situation where toxoplasmosis is of low probability (10 % probability). A negative test in the same clinical situation (10 % pretest probability) would exclude the diagnosis with 98.6 % confidence. While the results shown in Table (5) indicate the validity of parallel combination of the two tests (ELISA+ recomLine) in diagnosis of toxoplasmosis.

Table (5): The validity parameters for Positive serology (ELISA+recomLine) when used as a test to diagnose toxoplasmosis

	Final diagnosis of toxoplasmosis		
Serology (ELISA+recomLine)	Negative	Positive	Total
Negative(one or both negative)	10	2	12
Positive(Both positive)	0	8	8
Total	10	10	20

Sensitivity=80.0 %(44-96) ; Specificity=100.0 % ; Accuracy=90.0%(66-99) ; PPV at 10 % pretest probability =100.0 % ; PPV at 40 % pretest probability =100.0 % ; NPV at 10 % pretest probability =97.8 %

The sensitivity of combination of the two tests was 80.0%, while the specificity was assumed 100.0%, with 90% accuracy, the false negative results was 20.0 %. Positive tests can establish the diagnosis of toxoplasmosis with 100.0 % confidence in any clinical situation (at 40 % and 10 % pretest probability). These criteria of tests combination is assumed by the definition of diagnosed toxoplasmosis used in the present study. A negative test in low probability clinical situation (10% pretest probability) would exclude the disease with 97.8% confidence.

The results of RT-PCR that were used to diagnose toxoplasmosis were shown in Table (6).

Table (6): The validity parameters of real-time PCR when used as a test to diagnose toxoplasmosis

	Final diagnosis of toxoplasmosis		
Real-time PCR	Negative	Positive	Total
Negative	10	6	16
Positive	0	4	4
Total	10	10	20

Sensitivity=40.0%(14-73%); Specificity = 100.0 %;Accuracy= 70.0%(46-87%); PPV at 10 % pretest probability =100.0 %; PPV at 40 % pretest probability =100.0 %; NPV at 10 % pretest probability =93.8 % .Figures between brackets are 95% confidence interval for the estimation .

The sensitivity of RT-PCR was 40%, the specificity was assumed 100%, with 70 % accuracy. A positive test can establish the diagnosis of toxoplasmosis with 100 % in any clinical situation (40 % and in 10 % pretest probability). A negative test in the same clinical situation at 10% pretest probability would exclude the diagnosis with 93.8 % confidence.

Discussion:

Toxoplasmosis can vary from asymptomatic, self limiting infection to a fatal disease. Therefore toxoplasmosis is of great clinical importance in man in two major situations as seen in patients with congenital infection, since 5-24% of children becoming ill and dying during neonatal period (12), or in debilitated patients in whom underlying condition may influence the final outcome of the infection (13). Primary acquired infection during pregnancy may cause miscarriage, permanent neurological damage, premature birth & visual impairment (14,15,16,17,18,19).

The diagnosis of T.gondii infection in human can be determined by variable immunological and molecular methods. The current parasitological methods were used for the detection of the infection in the newborn or compromised host. Therefore, the methods dealing with the anti- T.gondii antibody, or circulating antigen, are more widely used. The methodology for the antibody detection is well established, allowing the identification of serologic profile, correlated with the stage of the infection (20). Real-time PCR has recently been introduced for the diagnosis of Toxoplasmosis (7,8). It combines the steps of amplification and PCR product detection in a single phase, thereby shortening the turnaround time from (24 to 48 hours) to less than 4 hours. Real-time PCR uses fluorescence–labeled oligonucleotide probe, which eliminates the need for post PCR product uses a processing (9). The Line immunoassay based on recombinant antigen (which is called recomeLine test kit) Toxoplasma is designed as a confirmation test on the basis of antigen sprayed onto nitrocellulose strips. The line assay technique facilitates detection and identification in a single test and at a single glance of IgG, IgM, or IgA antibodies against highly specific genetically engineered Toxoplasma antigens (2). In the present study the results show that the sensitivity of IgM –Toxoplasma antibodies detected by ELISA was 91.7%, also the sensitivity of recomLine Toxoplasma IgM assay was 90% .While using the combination system of ELISA and recomLine assays, which had 80% sensitivity that was higher to that sensitivity of real-time PCR in the present work was 40% which was within the 95% confidence interval of the present work sensitivity (16-73%), while (Kasper et al., 2009) conclude that real-time PCR has a good sensitivity(100%), which is slightly higher than the upper limit of 73% for 95% confidence interval of the sensitivity and this is because of very small sample size .The wide confidence interval shown

in the results of validity estimates, dose not under more value of present work, since it was the first Toxoplasma diagnosis of toxoplasmosis by real-time PCR and recomLine in Iraq . The results obtained make the final diagnosis of toxoplasmosis based on the new test system which combines using the parallel combination system of ELISA and recomLine assays. Since the sensitivity of combination of the two tests (ELISA and recomLine)assays was 80% which is slightly lower than the sensitivity of using each assay alone, which justify the use of combination, since it make the specificity perfect. The use of that combination system which includes ELISA that was used in a wide range for the diagnosis of toxoplasmosis was sensitive and available with low cost (21). Also, recomLine assay which use various recombinant antigens leading to give information on the specific antibodies at glance with allocated to the various stage of Toxoplasma status (3,,22). In addition recomLine assay offers the same advantages as the western blot (3). PCR is highly sensitive and specific and can detect 100 or less organism can be extracted in 200µlblood and can detected by real-time PCR , make this test is the test of choice , but care should be taken if it is the only test available(23). A more reliable diagnosis will be gained if it is used in combination with other diagnostic data (24). However real-time PCR is a highly sensitive and specific method, but it is expensive and requires specialized detection system and therefore may only be cost effective in laboratories where analysis of large numbers of samples is carried out (25). Since, the real-time PCR including an internal control, which can be used to detect low amount of T.gondii in a sample by amplification of part of the 529 bp element which is repeated with 200-300 folds in T.gondii genome (10). In general a reliable diagnosis of patients state regarding Toxoplasma infection can not be derived from a single examination, since it would be impossible to detect whether the antibodies concentration are currently on the rise or on the decrease (3). In addition to confirming infection, such as test can aid in determining progresses influence management and assist in monitoring response to treatment (21, 27).

Conclusion:

The new test system that use the parallel combination of (ELISA and recomLine assay), can be used in the diagnosis, with high sensitivity than that the sensitivity of RT-PCR. In addition these tests are available and low cost too and can be reliable to establish the diagnosis of acute toxoplasmosis.

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