Research Article

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Comparison of three immunoassays for detection of anti-toxoplasma IgM antibodies in pregnant women

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Abstract

Aim of investigation: Comparison of an electrochemiluminescence technique (ECL, applied in Cobas 6000 instrument), an enzyme-linked immunosorbent assay (ELISA, applied in CHORUS instrument) and an enzyme-linked fluorescent assay (ELFA, applied in MINI-VIDAS instrument) for early diagnosis of *Toxoplasma gondii* infection in pregnant women. Medical diagnosis is required to determine the most sensitive techniques to diagnose this pathogen, in the framework of which is developed this scientific work. This is very important due to the multiple fetal infections during pregnancy.
Methods: ECL, ELISA and ELFA techniques are used to detect anti-toxoplasma IgM antibodies in pregnant women, during the first trimester of pregnancy. 200 samples were analyzed with each technique and sensitivity and specificity are evaluated for each of them.
Results: ECL technique has resulted in higher sensitivity (100%) and specificity (99.3%), while ELISA technique has resulted in lower sensitivity (87.5%) and specificity (97.3%).
Conclusion: Analysis of the results confirmed the usefulness of ECL technique for an early diagnosis of *Toxoplasma gondii* infections in pregnant women. Anyway, for diagnostic purposes, the results should always be assessed in conjuction with the patient's medical history and other clinical examinations.

Keywords: Electrochemiluminescence; ELISA; ELFA; Toxoplasma gondii; sensitivity.

1. Introduction

Toxoplasma gondii is a well-known obligate intracellular protozoa pathogen of virtually all warm-blooded animals and commonly infects human worldwide (Beghetto et al. 2005). The infection is mainly acquired by ingestion of food or water that is contamined by mature oocysts shed by cats or by undercooked meat containing tissue cysts. Acute infection of toxoplasmosis in early pregnancy of women carries the peril of transmitting the infection to the fetus with serious and unpredictable consequences in later life (Beghetto et al. 2005; Many, 2006). A first step in diagnosing

acute primary congenital *Toxoplasma gondii* infections is most commonly made by the detection of anti-Toxoplasma-specific IgG and IgM antibodies. The assay of specific IgM is of great importance in the diagnosis of primary infection and samples being reactive for IgM antibodies indicate an acute, recent or reactivated infection (Van Helden, 2009). Medical diagnosis is working to determine the most sensitive techniques for the detection of anti-Toxoplasma gondii antibodies, in the framework of which is developed this scientific work.

2. Materials and Methods

200 pregnant women, in an age ranging from 18 to 40 years, who were retested for anti-toxoplasma IgM antibodies and have come out with a negative result in the preliminary testing, were involved in this sudy. Serum samples were analyzed with electrochemiluminescence assay (ECL, applied in COBAS 6000 instrument), enzyme-linked immunosorbent assay (ELISA, applied in CHORUS instrument) and enzyme-linked fluorescent assay (ELFA, applied in MINI-VIDAS instrument), including 150 negative and 50 positive samples for anti-toxoplasma IgM antibodies. Further, the results were used to build ROC curves and to calculate sensitivity and specificity (with SPSS and MedCalc), which consist of statistical measurements of quality of a test. In addition, these results are used to calculate the area under the ROC curve (AUC), which is a measure of how well a parameter can distinguish between two diagnostic assays.

2.1 Principle of Electrochemiluminescence technique for detection of anti-toxoplasma IgM antibodies

This technique is applied on Cobas 6000 instrument (Roche Diagnostics, 2011). The test principle is μ-Capture with a total duration of 18 minutes. **The first incubation:** 10 μL of sample are automatically prediluted 1:20 with Elecsys Diluent Universal. T. gondii-specific recombination antigen labeled with a ruthenium complex is added. Anti-Toxo IgM antibodies present in the sample react with the ruthenium-labeled T. gondii -specific recombination antigen.

The second incubation: Biotinylated monoclonal anti-h-IgM-specific antibodies and streptavidin-coated microparticles are added. The complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined automatically by the software by comparing the electrochemiluminescence signal obtained from the reaction product of the sample with the signal of the cutoff value previously obtained by Toxo IgM calibration.

Specimen type and collection: Human serum collected in separating tube gel. Samples can be stored at 2-8°C for up to 5 days; if longer storage is required, freeze

at -25° ± 6 C.

Description of the reagent: M: Streptavidin-coated-microparticles (transparent cap), 1 bottle, 6.5mL. R1: Toxoplasma-Ag-Ru$(bpy)_3^{2+}$ (gray cap), 1 bottle, 9 mL. R2: Anti-h-IgM-Ab-biotin (black cap), 1 bottle, 9 mL.

2.2 Principle of enzyme-linked immunosorbent assay for detection of anti-toxoplasma IgM antibodies

This test is applied on CHORUS instrument, which is a new device in medical diagnostics (DIESSE Diagnostica manual, 2011). The test is based on the ELISA principle. The partially purified toxoplasma antigen is bound to the solid phase. Through incubation with human serum diluted in a diluent which blocks the IgG, the specific IgM are bound to the antigen. After washing to eliminate the proteins which have not reacted, the sample is incubated with the conjugate composed of monoclonal anti-human IgM antibodies labelled with peroxidase. The unbound conjugate is eliminated and the peroxidase substrate is added. The colour which develops is proportional to the concentration of specific antibodies present in the serum. The disposable devices contain all the reagents to perform the test when applied on the CHORUS instrument. The control serum is used to check the validity of the results obtained. It should be used as reported in the operating manual. If the instrument signals that the control serum has a value outside the acceptable range, the calibration must be repeated. The previous result will be automatically corrected.

Description of Toxoplasma strip

The strip consist of 7 wells covered with a labelled, foil seal. The label comprises a bar code which mainly indicates the assay code, kit lot number and expiration date. The foil of the first well is perforated to facilitate the introduction of the undiluted sample. The wells in the center section of the strip contain the various reagents required for the assay:

Position 1: Empty well in which the operator must place the undiluted serum.
Position 2: Conjugate 0,35 ml.
Position 3: Diluent for the samples 0,35ml.
Position 4: TMB substrate 0,35 ml.
Position 5: Uncoated microplate well.
Position 6: Microplate well coated with purified Toxoplasma antigens.
Position 7: Empty.

Specimen type and collection

Human serum collected in separating tube gel in the normal manner from the vein and handled with all precautions. Samples can be stored at 2-8°C for 4 days, or frozen for longer periods at -20°C.

2.3. Principle of enzyme-linked fluorescent assay for detection of anti-toxoplasma IgM antibodies

This technique is applied in MINI-VIDAS instrument (BIOMERIEUX SA. Vidas Manual instrument). The assay principle combines a two step enzyme immunoassay

sandwich method with a final fluorescent detection. The solid phase receptacle (SPR) serves as the solid phase as well as the pipetting device. Reagents for the assay are ready to use and pre-dispensed in the sealed reagent strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times. Anti Toxoplasma IgM antibodies present in the serum will bind to the Toxoplasma antigen coating the anterior of the SPR. Unbound components are eliminated during the washing steps. An Alkaline phosphatase-labeled monoclonal anti-human IgM antibody is cycled in and out of the SPR. A final wash step removes unbound components. During the final detection step, the substrate (4-Methyl-umbelliferyl phosphate) is cycled in and out the SPR. The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methyl-umbelliferone), the fluorescence of which is measured at 450nm. At the end of the assay, results are automatically calculated by the instrument in relation to the calibration curve stored in memory, and then printed out. The interior of the SPR is coated during production with purified Toxoplasma antigen. Each SPR is identified by the TOXOM code. Before each new lot of reagents is used, specifications must be entered into the instrument using the master lot entry card. Calibration, using the standart provided in the kit, must be performed each time a new lot of reagents is opened, after the master lot data has been entered. Calibration should than be performed every 14 days. This operation provides instrument-specific calibration curves and compensates for possible minor variation in assay signal. One positive control and one negative control are included in each VIDAS TOXOM kit. These controls must be performed immediately after opening a new kit to ensure that reagent performance has not been altered. Each calibration must also be checked using these controls. Results can not be validated if the control values deviate from the expected values.

Description of the Toxoplasma IgM (TOXOM) Strip

The strip consist of 10 wells covered with a labelled, foil seal. The label comprises a bar code which mainly indicates the assay code, kit lot number and expiration date. The foil of the first well is perforated to facilitate the introduction of the sample. The last well of each strip is a cuvette in which the fluorometric reading is performed. The wells in the center section of the strip contain the various reagents required for the assay.

Specimen Type and Collection

Human serum collected in separating tube gel collected in the normal manner from the vein. Samples can be stored at 2-8°C for up to 5 days; if longer storage is required, freeze at -25° ± 6 C.

3. Results and Discussion

Results of anti-toxoplasma IgM antibodies measurements are summarized in table 1.
Table 1: Results of ECL, ELISA and ELFA immunoassays for detection of anti-toxoplasma IgM antibodies.

Samples	ECL (COBAS 6000) TOXO M		
	Positive	Doubtful	Negative
(Positive) N=50	50	0	0
(Negative) N=150	1	1	148
	ELISA (CHORUS) TOXO M		
(Positive) N=50	35	10	5
(Negative) N=150	4	2	144
	ELFA (MINI-VIDAS) TOXO M		
(Positive) N=50	43	3	4
(Negative) N=150	2	2	146

Results of sensitivity and specificity of ECL, ELISA and ELFA immunoassays for detection of anti-toxoplasma IgM antibodies measurements are summarized in table 2.

Table 2: Results of sensitivity and speificity of ECL, ELISA and ELFA immunoassays for detection of anti-toxoplasma IgM antibodies.

Assay	Sensitivity (%)	Specificity (%)
ECL (COBAS 6000) TOXO M	100	99.3
ELISA (CHORUS) TOXO M	87.5	97.3
ELFA (MINI-VIDAS) TOXO M	91.5	98.6

The ECL immunoassay resulted with high sensitivity and specificity 100% and 99,3 % respectively, for the detection of anti-toxoplasma IgM antibodies. These high values of sensitivity and specificity of the COBAS 6000 system and ECL technique, are attributed to some important features of this system (Prusa et al. 2010): the use of two-dimensional barcode on all reagents to minimize possible errors and maintaining constant control of the calibration curve (Roche Diagnostics, 2011).
The ELISA immunoassay resulted with lower sensitivity and specificity of 87,5% and 97,3% for the detection of anti-toxoplasma IgM antibodies. The CHORUS instrument expresses the result as an index (ratio between the OD value of the test sample and that of the cutoff) which can be used as a quantitative measure, as it is proportional to the amount of specific IgM present in the sample (DIESSE, 2011). The results of the assay must be interpreted with caution and in conjuction with information available from the clinical evaluation and other diagnostic data. Sera from patients in an early or late stage of the disease could give a repeatedly negative result close to the cut-off value (Hierl, 2004; Van Helden, 2009). In such cases, a confirmation of the result is recommended. Also, all positive test results require careful interpretation since false positive reactions or heterotypic IgM responses may occur with sera from patients

with Varicella Zoster (Bastien, 2007; Berth, 2010; Van Helden, 2009).

In the diagnosis of pregnant women, especially in the first trimester of pregnancy, lack of false negative results for IgM antibodies *Toxoplasma gondii* is very important. This relates to the fact that the existence of false negative results is associated with lack of diagnosis and treatment of acute infections in the fetus and newborn (Bastien, 2007; Bessieres et al. 2009; Prusa, 2012; Sterkers et al. 2009).

Fals positive, fals negative and doubtful results are related to the fact that samples in the initial phase of an acute infection, may not show detectable levels of anti-toxoplasma IgM antibodies. For this reason the detection of antibodies against *Toxoplasma gondii* in a single sample is not enough (Jost et al. 2011; Van Helden, 2009; Sterkers et al. 2009). Also, interference may occur in some samples containing antibodies against components of the reagents, or in some patients specific anti-toxoplasma IgM antibodies may return to nonreactive levels within weeks (Buffolano et al. 2005; Van Helden, 2009).

Another reason may be the fact that there is a non specific glycolipid antigen for *Toxoplasma gondii*, which operates in a cross-reaction with antigens of different origins, resulting in false positive, false negative and doubtful results (Van Helden, 2009).

The evaluation of the results showed a good concordance between the three immunoassays for the detection of anti-toxoplasma IgM antibodies.

1. ECL-ELISA: r=0,839, (p< 0.01). These results are expressed in the following scatterplot (Fig. 1). Also, ANOVA analysis showed a non-signifikative difference between the two immunoassays: F0.05[1,398] = 0,665, p = 0.415 (α=0.05).

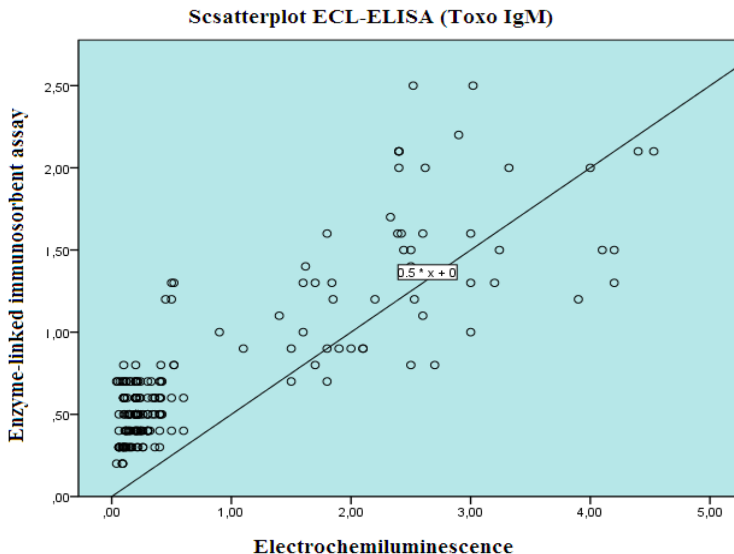

Fig 1. Scatterplot analysis of 200 sera measured by electrochemiluminescence assay (ECL-IgM) and enzyme-linked immunosorbent assay (ELISA-IgM) for detection of

anti-toxoplasma IgM antibodies. The linear line is characterized by y=0.5*x equation. The scatterplot is linear, positive with few values that deviate the linear line. This means that ECL and ELISA immunoassays have a good concordance for detection of anti-toxoplasma IgM antibodies.

2. ECL-ELFA: r=0,882, ($p < 0.01$). These results are expressed in the following scatterplot (Fig. 2). Also, ANOVA analysis showed a non-signifikative difference between the two immunoassays: F0.05[1,398] = 2.622, p = 0.106 (α=0.05).

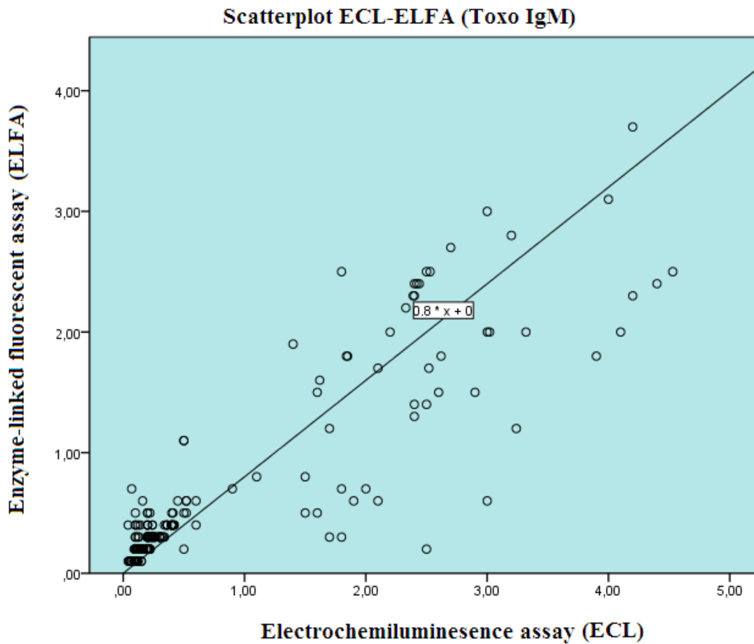

Fig 2. Scatterplot analysis of 200 sera measured by electrochemiluminescence assay (ECL-IgM) and enzyme-linked fluorescent assay (ELFA-IgM) for detection of anti-toxoplasma IgM antibodies. The linear line is characterized by y=0.8*x equation. The scatterplot is linear, positive with few values that deviate the linear line. This means that ECL and ELFA immunoassays have a good concordance for detection of anti-toxoplasma IgM antibodies.

3. ELISA-ELFA: r=0.839, ($p < 0.01$). These results are expressed in the following scatterplot (Fig. 3). Also, ANOVA analysis showed a non-signifikative difference between the two immunoassays $F_{0.05[1,398]} = 1.691$ (p = 0.194) (α=0.05).

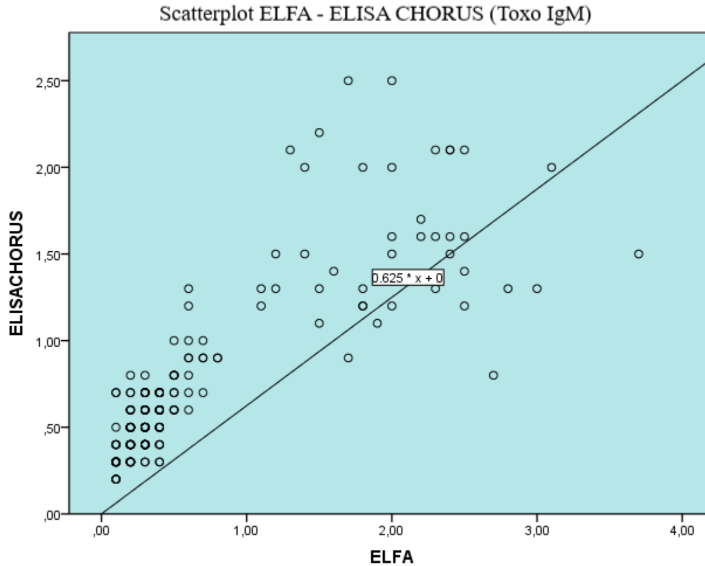

Fig 3. Scatterplot analysis of 200 sera measured by enzyme-linked fluorescent assay (ELFA-IgM) and enzyme-linked immunosorbent assay (ELISA-IgM) for detection of anti-toxoplasma IgM antibodies. The linear line is characterized by y=0.625*x equation. The scatterplot is non linear, positive with few values that deviate the linear line. This means that ELFA and ELISA immunoassays have a good concordance for detection of anti-toxoplasma IgM antibodies.

The receiver operating characteristics (ROC) curves were generated for these techniques (Fig. 4).

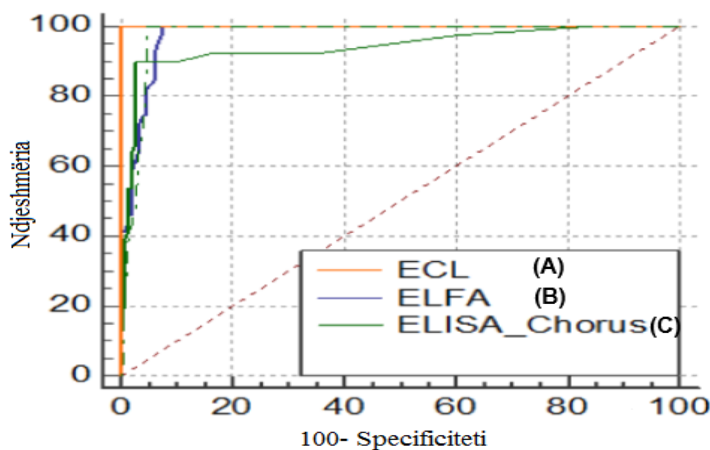

Fig 4. ROC Curves for comparison of electrochemiluminescence, enzyme-linked fluorescent assay and enzyme-linked-immunosorbent assay for detection of anti-toxoplasma IgM antibodies. (A) Shows the ROC curve of electrochemiluminescence

assay with an area under the ROC curve (AUC) of 1,00. (**B**) Shows the ROC curve of enzyme-linked-fluorescent assay with an AUC of 0.974; (**C**) Shows the ROC curve of enzyme-linked- immunosorbent assay with an AUC of 0.941.

The ROC curve is a fundamental tool as diagnosis for test evaluation. In a ROC curve the true positive rate (Sensitivity) is plotted in function of the false positive rate (100-Specificity) for different cut-off points of a parameter. Each point represents a sensitivity/specificity pair corresponding to a particular decision threshold (Kumar et al. 2011). The ROC area for electrochemiluminescence assay was higher than those for enzyme-linked-immunosorbent assay and enzyme-linked fluorescent assay. No statistically significant differences in ROC curves were noted between electrochemiluminescence assay, enzyme-linked-immunosorbent assay and enzyme-linked fluorescent assay for detection of anti-toxoplasma IgM antibodies in pregnant women.

The three imunoassays resulted with AUC> 0.5. The ECL immunoassay resulted with high AUC (1,000) for the detection of anti-toxoplasma IgM antibodies, while ELISA immunoassay resulted with lower AUC (0,941) for the detection of anti-toxoplasma IgM antibodies. These results are expressed in ROC curves, (Fig. 4).

In conclusion, analysis of the results revealed a good level of concordance between the three immunoassays and confirmed the usefulness of electrochemiluminescence assay to diagnose acute *Toxoplasma gondii* infections in pregnant women. Anyway, for diagnostic purposes, the results should always be assessed in conjuction with the patient's medical history and other clinical examinations.

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