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Evaluation of an electrochemiluminescence immunoassay and an enzyme-linked fluorescent assay for detection of anti-cytomegalovirus IgM and anti-toxoplasma IgM antibodies in pregnant women

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Abstract— **Nowdays, it is noticed an increase in morbidity from infectious factors, among which the principal ones are viral, bacterial and parasitic infections. This is quite sensitive in pregnant women, whose infections, especially in the first trimester of pregnancy cause malformation of the fetus that is being formed. This is more complicated in cases of *Toxoplasma gondii* and *Cytomegalovirus* because of cross reactions of their antibodies against similar antigenic epitopes. For this reason the aim of this study was the detection of gestational *Cytomegalovirus* and *Toxoplasma gondii* infections. Cytomegalovirus (CMV) is a herpes virus transmitted by intimate contact with infected excretions such as saliva, urine, cervical and vaginal excretions, semen, breast milk and blood. *Toxoplasma gondii* is a parasitic protozoa which can be transmited by eating infected meat or from mother to fetus during the first trimester of pregnancy. Because diagnosis of maternal infections solely depends on serology, routine tests with high sensitivity and specificity are required. Medical diagnostic is working to determine the most sensitive techniques for the detection of *anti-cytomegalovirus* IgM and *anti-toxoplasma* IgM antibodies, in the framework of which is developed this scientific work. This study compares an electrochemiluminescence immunoassay (ECL, applied in COBAS 6000 instrument) with an enzyme-linked fluorescent assay (ELFA, applied in MINI-VIDAS instrument) for detection of *anti-cytomegalovirus* IgM and *anti-toxoplasma* IgM antibodies in pregnant women. 400 pregnant women were involved in the study and serum samples were analyzed with both techniques. Sensitivity and specificity were evaluated and ECL immunoassay resulted with high sensitivity and specificity (98% - 100%), while ELFA immunoassay resulted with lower sensitivity and specificity (89,4% - 98,6%). The evaluation of the results showed a good concordance between the two immunoassays, but at the same time a better performance of ECL immunoassay as a first-line screening method to detect gestational *Cytomegalovirus* and *Toxoplasma gondii* infections. Anyway, for diagnostic purposes, the results should always be assessed in conjuction with the patient's medical history and other clinical examinations.**

Keywords— Cytomegalovirus; Toxoplasma gondii; Electrochemiluminescence; enzyme-linked fluorescent assay; sensitivity; specificity.

I. INTRODUCTION

Cytomegalovirus, a member of the herpes virus family, is ubiquitous in all human populations, causing infections which are followed by life-long latency in the host with occasional reactivations as well as recurrent infections. Transmission of infection requires intimate contact with infected excretions such as saliva, urine, cervical and vaginal excretions, semen, breast milk and blood. However, primary maternal CMV infection during pregnancy carries a high risk of intrauterine transmission which may result in severe fetal damage, including growth and mental retardation, jaundice and CNS abnormalities [11], [13]. *Toxoplasma gondii* is a well-known obligate intracellular protozoa pathogen of virtually all warm-blooded animals and commonly infects human worldwide. 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 [9], [12]. A first step in diagnosing acute primary congenital *Cytomegalovirus* and *Toxoplasma* infections is most commonly made by the detection of anti-CMV-specific IgG and IgM antibodies or 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 [11], [13]. Medical diagnostic is working to determine the most sensitive techniques for the detection of antibodies against *Cytomegalovirus* and *Toxoplasma gondii*, in the framework of which is developed this scientific work.

II. MATERIALS AND METHODS

200 pregnant women, with age ranging from 18 to 40 years, who are retested for anti-cytomegalovirus IgM antibodies and 200 other pregnant women who are 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) and enzyme-linked fluorescent assay (ELFA, applied in MINI-VIDAS instrument), including 150 negative and 50 positive samples, respectively, for anti-cytomegalovirus IgM and anti-toxoplasma IgM antibodies. Further, the results were used to build ROC curves and to calculate sensitivity and specificity (with SPSS and MedCalc), which are 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.

A. Principle of Electrochemiluminescence technique for detection of anti-cytomegalovirus IgM antibodies

This technique is applied on Cobas 6000 instrument. 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. Biotinylated monoclonal anti-h-IgM-specific antibodies are added. The second incubation: CMV-specific recombination antigen labeled with a ruthenium complex and streptavidin-coated microparticles are added. Anti-CMV IgM antibodies present in the sample react with the ruthenium-labeled CMV-specific recombination antigen. 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 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 CMV IgM calibration.

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

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.

B. Principle of Electrochemiluminescence technique for detection of anti-toxoplasma IgM antibodies

This technique is applied on Cobas 6000 instrument. 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.

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.

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.

C. Principle of enzyme-linked fluorescent assay for detection of anti-cytomegalovirus IgM antibodies

This technique is applied in MINI-VIDAS 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 CMV IgM antibodies present in the serum will bind to the CMV 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 CMV antigen. Each SPR is identified by the CMVM 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 CMVM 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 Cytomegalovirus IgM (CMVM) 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.

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

This technique is applied in MINI-VIDAS 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.

III. RESULTS AND DISCUSSION

Results of anti-cytomegalovirus IgM and anti-toxoplasma IgM antibodies measurements are presented in summary in table 1.

TABLE 1: RESULTS OF ECL AND ELFA IMMUNOASSAYS FOR DETECTION OF ANTI-CYTOMEGALOVIRUS IGM AND ANTI-TOXOPLASMA IGM ANTIBODIES

Samples	**ECL (COBAS 6000) CMV M**		
	Positive	**Doubtful**	**Negative**
(Positive) N=50	49	0	1
(Negative) N=150	2	0	148
	ELFA (MINI-VIDAS) CMV M		
(Positive) N=50	42	3	5
(Negative) N=150	3	3	144
	ECL (COBAS 6000) TOXO M		
(Positive) N=50	50	0	0
(Negative) N=150	1	1	148
	ELFA (MINI-VIDAS) TOXO M		
(Positive) N=50	43	3	4
(Negative) N=150	2	2	146

Results of sensitivity and specificity of ECL and ELFA assays for detection of anti-cytomegalovirus IgM and anti-toxoplasma IgM antibodies measurements are presented in summary in table 2.

TABLE 2: RESULTS OF SENSITIVITY AND SPEIFICITY OF ECL AND ELFA IMMUNOASSAYS FOR DETECTION OF ANTI-CYTOMEGALOVIRUS IGM AND ANTI-TOXOPLASMA IGM ANTIBODIES

Assay	Sensitivity (%)	Specificity (%)
ECL (MINI-VIDAS) CMV M	98	98.7
ELFA (MINI-VIDAS) CMV M	89.4	98
ECL (MINI-VIDAS) TOXO M	100	99.3
ELFA (MINI-VIDAS) TOXO M	91.5	98.6

The evaluation of the results showed a good concordance between the two immunoassays: r=0,942, ($p< 0.01$) for the detection of anti-cytomegalovirus IgM antibodies and r=0,882, ($p< 0.01$) for the detection of anti-toxoplasma IgM antibodies. These results are expressed in the following scatterplots (Fig. 1, and Fig. 2,). Also, ANOVA analyse showed a non-signifikative difference between the two immunoassays: $F_{0.05[1,398]} = 0.111$, $p = 0.740$ ($\alpha=0.05$) for the detection of anti-cytomegalovirus IgM antibodies and $F_{0.05[1,398]} = 2.622$, $p = 0.106$ ($\alpha=0.05$) for the detection of anti-toxoplasma IgM antibodies.

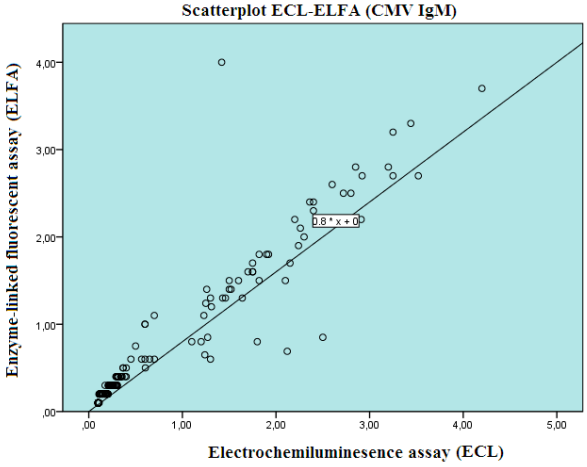

Fig. 1. Scatterplot analysis of 200 sera measured by electrochemiluminescence assay (ECL-IgM) and enzyme-linked fluorescent assay (ELFA-IgM) for detection of anti-cytomegalovirus IgM antibodies. The linear line is characterized by y=0.8*x equation. The scatterplot is nonlinear, positive with few values that deviate the linear line. This means that ECL and ELFA immunoassays have a good concordance for detection of anti-cytomegalovirus IgM antibodies.

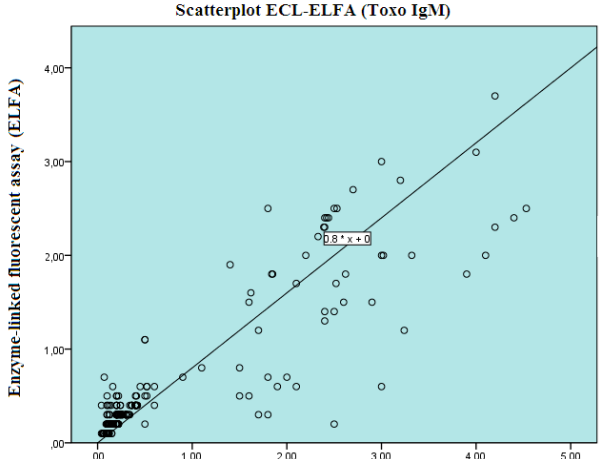

Fig. 2. Scatterplot analysis of 200 sera measured by electrochemiluminescence assay (ECL-IgM) and enzyme-linked fluorescent assay (ELFA-IgM) for detection of anti-toxoplasma IgM antibodies. The linear line is characterized by y=0.8*x equation. The scatterplot is linear, positive with few values that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

A receiver operating characteristics (ROC) curve was generated for each antibody tested (Fig. 3, and Fig.4,).

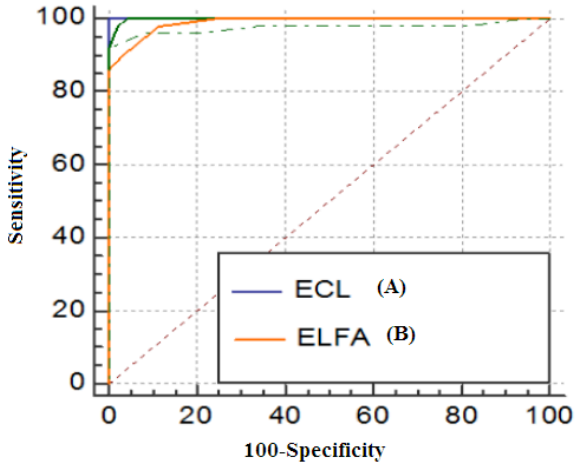

Fig. 3. ROC Curves for comparison of electrochemiluminescence assay and enzyme-linked-fluorescent assay for detection of anti-cytomegalovirus IgM antibodies. (**A**) Shows the ROC curve of electrochemiluminescence assay with an area under the ROC curve (AUC) of 1,00. (**B**) Shows the ROC curve of enzyme-linked-fluorescent assay with an AUC of 0.99.

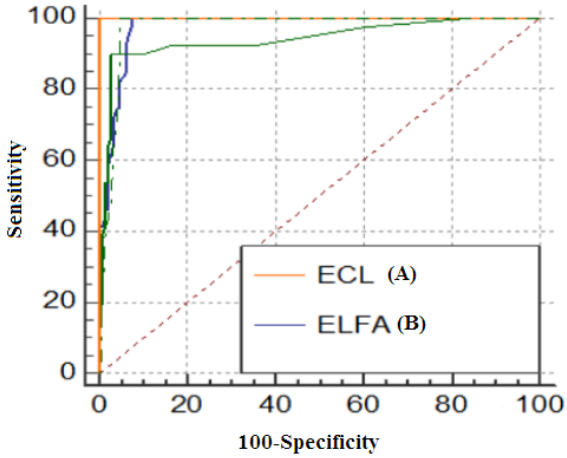

Fig. 4. ROC Curves for comparison of electrochemiluminescence assay and enzyme-linked-fluorescent assay for detection of anti-toxoplasma IgM antibodies. (**A**) Shows the ROC curve of electrochemiluminescence assay with an area under the ROC curve (AUC) of 1,00. (**B**) Shows the ROC curve of enzyme-linked-fluorescent assay with an AUC of 0.974.

The ROC curve is a fundamental tool for diagnostic 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 [5]. The ROC area for electrochemiluminescence assay was higher than those for enzyme-linked-fluorescent assay. No statistically significant differences in ROC curves were noted between electrochemiluminescence assay and enzyme-linked-fluorescent assay for detection of anti-cytomegalovirus IgM and anti-toxoplasma IgM antibodies in pregnant women.

The two imunoassays resulted with AUC> 0.5. The ECL immunoassay resulted with high AUC (1,000) for the detection of anti-cytomegalovirus IgM antibodies and anti-toxoplasma IgM antibodies, while ELFA immunoassay resulted with lower AUC (0,990) for the detection of anti-cytomegalovirus IgM antibodies and 0,974 for the detection of anti-toxoplasma IgM antibodies. These results are expressed in ROC curves, (Fig. 3, and Fig.4,).

The ECL immunoassay resulted with high sensitivity and specificity of 98% and 98,7%, respectively, for the detection of anti-cytomegalovirus IgM antibodies and 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: the use of two-dimensional barcode on all reagents to minimize possible errors and maintaining constant control of the calibration curve.

The ELFA immunoassay resulted with high sensitivity and specificity of 89,4% and 98%, respectively, for the detection of anti-cytomegalovirus IgM antibodies and 91,5% and 98,6%, respectively, for the detection of anti-toxoplasma IgM antibodies. The MINI-VIDAS instrument expresses the result as an index (ratio of the fluorescent signal found for the serum to be tested, over the standart signal stored in the memory). Fluorescence is measured twice in the reagent strip reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced onto the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The RFV (Relative Fluorescence Value) is calculated by substracting the background reading from the final result. Interference may be encountered with certain sera containing antibodies directed against reagent components. For this reason, assay results should be interpreted as part of a complete clinical profile.

In the diagnosis of pregnant women, especially in the first trimester of pregnancy, lack of false negative results for IgM antibodies to *Cytomegalovirus* and *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 baby [8], [10].

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-cytomegalovirus IgM and anti-toxoplasma IgM antibodies. For this reason, the detection of antibodies against *Cytomegalovirus* and *Toxoplasma gondii* in a single sample is not enough [14] [15]. Also, interference may occur in some samples containing antibodies against components of the reagents, or in some patients, specific anti-cytomegalovirus IgM antibodies may return to nonreactive levels within weeks after infection with cytomegalovirus[14] [15].

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 fals positive, fals negative and doubtful results [9]. Also samples of patients who suffer from primary infections of Epstein-Barr virus may result positive for anti-cytomegalovirus IgM antibodies. This may be related to the fact that both viruses are in the same family of herpesviruses [15].

In conclusion, the evaluation of the results showed a good concordance between the two immunoassays, but at the same time a better performance of ECL immunoassay as a first-line screening method to detect gestational *Cytomegalovirus* and *Toxoplasma gondii* infections. 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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