Automated Time-resolved Immunofluorometric Assay for *Toxoplasma gondii*-specific IgM and IgA Antibodies: Study of More Than 130 000 Filter-Paper Blood-Spot Samples from Newborns

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**Background:** To screen for congenital toxoplasmosis, we developed a time-resolved immunofluorometric assay for the simultaneous detection of *Toxoplasma gondii*-specific IgM and IgA in filter-paper samples collected from newborns 4–7 days after birth.

**Methods:** The assay was performed on the Auto-DELFIATM, and results were calculated based on the ToxoM WHO Third International Reference Serum. Comparison with an in-house μ-capture immunoassay was carried out retrospectively on filter-paper samples from children with confirmed congenital toxoplasmosis. Prospectively the assay was compared with a μ-capture immunoassay on 68 394 samples and a commercially available assay on another 69 467 samples. Before serum was requested from the newborn, positive samples were tested for IgA and IgM separately and in an IgM-immunosorbent agglutination assay developed for filter-paper samples.

**Results:** Intra- and interassay variations (CVs) were 8% and 16%, respectively. The cutoff of 5 units/mL produced a 0.5% retest rate. The assay detected 13 of 18 (72%) samples from newborns diagnosed with congenital toxoplasmosis in the retrospective study. Prospectively, the assay identified 24 newborns who were later diagnosed with congenital toxoplasmosis. Results for all 24 cases were positive by the respective comparison method. No cases were detected solely by the IgA antibodies in the sample.

**Conclusion:** Neonatal screening for congenital toxoplasmosis can be automated by use of purified europium-labeled antigen for detection of *T. gondii*-specific IgM and IgA eluted from filter-paper samples.

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In Denmark, 75% of all pregnant women are seronegative for, and thus susceptible to infection with, toxoplasmosis. The frequency of *Toxoplasma* infection in pregnant women is 0.29%, and the transmission rate is 19.4% (1). Infection of the fetus early in pregnancy may lead to severe clinical manifestations, e.g., chorioretinal lesions and intracranial calcifications, in the child (2, 3). The risk of infection of the fetus increases as the pregnancy progresses, but the vulnerability of the fetus decreases (2, 4). The majority of newborns with congenital toxoplasmosis (CT)3 are asymptomatic and may develop symptoms as they grow older (1, 3). If infected newborns are detected in a screening program and treatment is instituted, the prognosis is much better than if they are diagnosed later in life with overt clinical disease (5–7).

Infection by the parasite induces *Toxoplasma*-specific IgM and IgA antibodies followed some days later by a long-lasting specific IgG response. However, maternal IgG antibodies from a previous infection will be present in the newborn’s blood because IgG crosses the placenta. On the other hand, IgM and IgA do not cross the placenta and are used as markers of fetal infection during pregnancy (8–12). Wallon et al. (8) found varying sensitivity of
Toxoplasma-specific IgM and IgA in newborns for the detection of CT, depending on the time of maternal infection. Toxoplasma-specific IgM was found in 40% of infected newborns whose mothers were infected during the first and second trimesters after treatment in pregnancy. The sensitivity increased to 70% for mothers infected in the third trimester. The sensitivity of IgA antibodies was 0%, 60%, and 64% in newborns with mothers who seroconverted in the first, second, and third trimester, respectively. The aim of this study was to develop an efficient, automated assay for detection of Toxoplasma-specific IgM and IgA antibodies in filter-paper blood spots from newborns. In addition, to evaluate the technical performance of this time-resolved immunofluorometric assay (TRIFMA) as a screening method by comparison with a μ-capture immunoassay (1, 10) and the Neonatal Toxoplasma gondii IgM fluorometric enzyme immunocapture assay (FEIA; Labsystems).

Materials and Methods

All three assays use the SAG1 (previously P30) antigen to determine specificity of the captured antibodies. SAG1 is the major surface antigen of the tachyzoite stage of T. gondii (13). Briefly, the differences between the assays are as follows: The TRIFMA captures human IgM and IgA antibodies eluted from the filter-paper blood spots simultaneously. Labeled, purified SAG1 antigen is used to detect the Toxoplasma-specific IgM and IgA antibodies. The antigen-antibody complex is measured by time-resolved fluorometry (14). In the μ-capture immunoassay and the FEIA, IgM is eluted and immobilized by anti-human IgM. The Toxoplasma-specific IgM antibodies are detected with disrupted tachyzoites, followed by a monoclonal antibody specific for SAG1. The final step in the μ-capture assay involves addition of conjugated rabbit anti-mouse antibody, followed by substrate, and measurement by spectrophotometry at 405 nm. Measurement in the FEIA is by fluorometry (15).

Filter-paper samples giving a result above the cutoff in any of the three assays were further analyzed for IgM by use of an IgM-immunosorbent agglutination assay (ISAGA). In this assay, IgM eluted from the filter-paper samples is captured on anti-IgM wells of U-bottomed (ISAGA). In this assay, IgM eluted from the filter-paper blood spots in the Feia is by fluorometry at 405 nm. Measurement by spectrophotometry at 405 nm. Measurement in the FEIA is by fluorometry (15).

Filter-paper samples giving a result above the cutoff in any of the three assays were further analyzed for IgM by use of an IgM-immunosorbent agglutination assay (ISAGA). In this assay, IgM eluted from the filter-paper samples is captured on anti-IgM wells of U-bottomed microtiter strips, and specificity for Toxoplasma is detected by addition of formalin-fixed tachyzoites. A positive sample captures the parasites, whereas negative samples allow the parasite cells to settle and form a button. Conventional methods were used to confirm the diagnosis on serum requested from screen-positive children (16).

Samples

Filter-paper samples (PKU cards) previously tested for CT were obtained from the PKU Bio Bank (Statens Serum Institut), where they were stored at −20 °C. These samples were identified in a pilot screening study performed from 1992 to 1996 (1). Sixty randomly chosen samples matched for storage time were also retrieved. We tested 137 861 fresh filter-paper samples from the routine neonatal screening program in a prospective setting.

EUROPIUM-LABELED TOXOPLASMA ANTIGEN (TRACER)

Tachyzoites cultured in vero cells (10^8/mL) were mixed with an equivalent volume of 1% Igepal (cat. no. I3021; Sigma) in a 50 mmol/L Tris-HCl buffer (pH 8.0), followed by overnight incubation at room temperature. After centrifugation at 20 000g for 30 min, the supernatant was extensively dialyzed against a 0.15 mol/L phosphate-buffered saline solution (PBS). A 500-μL portion of the dialyzed solution was applied to a NAP5 column (Amersham Pharmacia Biotech) saturated with labeling buffer (0.1 mol/L NaHCO_3 and 0.15 mol/L NaCl, pH 8.5). Fractions were collected, and the absorbance was measured at 280 nm. Fractions containing protein were pooled and incubated overnight with 0.4 mg of europium labeling reagent (PLS-Wallac) at room temperature. After a buffer exchange to PBS on a PD10 column according to the manufacturer's instructions (Amersham Pharmacia Biotech), the protein-containing fractions were pooled and concentrated in a Centricon 100 (Amicon) with a cutoff of 100 kDa, according to the manufacturer's instructions. The final product (1 mL) was stored at 4 °C.

HPLC

A HPLC gel-filtration column (150 gfs; Amersham Pharmacia Biotech) was equilibrated with PBS at room temperature and operated at a flow rate of 0.5 mL/min. We applied 250 μL of labeled antigen, taken after PD10 elution, to the column and collected 0.5-mL fractions. The fractions were pooled and tested as tracers in the Toxoplasma anti-IgM assay.

SODIUM DODECYL SULFATE–POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOT

T. gondii antigen was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; non-reducing conditions) and transferred to 0.45 μm nitrocellulose membranes for immunoblotting as described previously (17).

Calibration Curve and Assay Controls

Red blood cells were produced by centrifuging blood collected in EDTA tubes (VT-100TK; Terumo Europe NV) at 2600g, followed by three washes with PBS.

An ampoule of WHO (ToxoM) Third International Reference Serum (lyophilized) was dissolved in 1 mL of water, giving a potency of 3000 units/mL IgM. For the calibration curve, this solution was diluted in PBS, and a twofold titration curve was constructed. After addition of washed red blood cells, the calibrators had concentrations of 150, 75, 37.5, 18.75, 9.375 and 0 units/mL and a hematocrit of 55%, measured in capillary tubes according to the manufacturer’s instructions (BRAND GmbH & Co.). The result was measured on a Mikro-Hämatokrit
According to instructions (Heraeus Sepatech). The calibrators were spotted on filter paper (no. 2992; Schleicher & Schuell). The filter paper was air dried and stored at −20 °C.

A positive control was produced from a pool of *Toxoplasma* IgM-positive serum, diluted with PBS, and red blood cells. The control had a hematocrit of 55%. A negative assay control consisted of PBS mixed with red blood cells at a hematocrit of 55%.

**Assay for toxoplasma-specific IgM and IgA antibodies (TOXO IgM+A assay)**

Microtiter plates (Nunc) were coated with 150 μL/well rabbit anti-human IgM and IgA antibodies (antibodies A0425 and A0262, respectively; Dako) diluted 500-fold in 0.1 mol/L carbonate buffer (pH 9.6). After overnight incubation at 4 °C, plates were washed once (Delfia Wash Solution; PLS-Wallac). Drying buffer consisting of 15 g/L bovine serum albumin (cat. no. A4503; Sigma) and 25 g/L sucrose dissolved in PBS was added to each well (200 μL/well). The plates were incubated for 30 min at room temperature, aspirated, and dried at room temperature for 3 h before being sealed with tape and stored at 4 °C.

After removal of the sealing tape, 3.2-mm discs were punched directly from the filter-paper blood samples into the wells with use of a DBS puncher (PLS-Wallac). The punched discs were eluted from the filter-paper blood samples into 50 μL of buffer (150 μL/well). The AutoDELFIA™ was programmed to do the following: Assay buffer (150 μL) was dispensed into each well. Assay buffer consisted of 15 g/L casein dissolved in PBS. After 2 h of incubation at 25 °C followed by removal of the discs and six washes with Wash Solution, 150 μL of tracer diluted 100-fold in assay buffer was added to each well. The plates were incubated for 2 h at 25 °C and washed six times, and 200 μL of Enhancement Solution was added to each well (PLS-Wallac). After counting, the results were calculated with Multicalc (PLS-Wallac) using the calibration curve produced from the third International WHO Reference Serum.

**Assay for toxoplasma-specific IgM or IgA antibodies**

For the assay for *Toxoplasma*-specific IgM or IgA antibodies, the same procedure as described above was followed, but the microtiter plate was coated with either rabbit anti-IgM or rabbit anti-IgA diluted 500-fold in the carbonate coating buffer.

**μ-capture immunoassay for toxoplasma-specific IgM antibodies**

Discs (3.2 mm) from the filter-paper blood samples were punched into microtiter wells coated with rabbit anti-human IgM (Dako). After elution of the discs, the *Toxoplasma*-specific IgM was detected with disrupted tachyzoites followed by incubation with a monoclonal antibody (S13) that recognizes the immunodominant surface antigen SAG1. A color reaction was obtained after incubation with alkaline phosphatase-conjugated rabbit anti-mouse antibody followed by substrate. The absorbance was measured at 405 nm (1, 10).

**IgM FEIA**

IgM FEIA reagent sets were purchased from Labsystems (product no. 6199802), and the instructions for use were followed.

**ISAGA ON FILTER-PAPER SPOTS**

Filter paper spots (3.2 mm) were eluted in 50 μL of buffer consisting of 10 g/L bovine serum albumin and 0.05 mL/L Triton X-100 (Sigma) dissolved in PBS. The samples were incubated for 2 h at 37 °C. Assay instructions from the manufacturer (cat. no. 75361; BioMérieux) were followed, and the results were read visually and reported as either positive or negative.

**Results**

The europium-labeled antigen was subjected to HPLC gel filtration as shown in Fig. 1. The fractions containing protein were pooled and tested against a calibration curve in the *Toxoplasma* IgM assay (Fig. 2). The best performing antigen eluted in the void volume (apparent molecular mass >150 kDa). When the antigen was purified by SDS-PAGE followed by Western blotting, the labeled antigen appeared as three bands at 28, 30, and 32 kDa. All three bands stained positive with the monoclonal antibody S13, which recognizes the antigen SAG1 (Fig. 3). All three bands also stained positive with serum from a patient with clinical toxoplasmosis (Fig. 4), whereas none of the bands stained positive with a serum pool from uninfected individuals (not shown). Several bands in the prelabeling solution assayed by SDS-PAGE with Western blotting were detected with the positive serum. These bands were not seen in the purified solution. The 28-kDa band was seen both in the prelabeling solution and in the purified solution.
Filter-paper samples from a pilot study performed from 1992 to 1996 (1) were tested in the preliminary AutoDELFIA assay. The samples had previously been tested in the /H9262/H9262-capture immunoassay for Toxoplasma-specific IgM antibodies. Serum corresponding to the filter-paper samples had been tested with the Platelia assay (Sanofi-Pasteur Diagnostics) to detect Toxoplasma-specific IgA. In the TOXOIgM/H11001/H11001-A AutoDELFIA assay, all the samples positive in the pilot study showed significantly increased counts (P < 10^{-6}) compared with 60 randomly selected filter-paper samples that were presumed negative (Fig. 5). When the counts were calculated as a percentage of the positive control, which was run in each assay, the two assays correlated very well (Fig. 6). Filter-paper samples were also tested in the Toxoplasma IgA assay, and again the positive samples were significantly increased (P = 0.0001) compared with the samples that were presumed negative (Fig. 7).

The final TOXO IgM+A assay was run on 68 394 samples from the neonatal screening program in parallel with the routine /H9262/H9262-capture assay and on 69 467 samples in parallel with the FEIA (15). PBS was used to dilute calibrators before they were applied to the filter paper, ensuring a reading above the lowest calibrator for most of the negative samples. This is necessary to calculate a correct cutoff in newborns with low background reactivity. The intra- and interassay variations (CVs), calculated using the positive control, were 8% and 16%, respectively. Samples were tested singly, and a cutoff of 5 units/mL was chosen because it gave an acceptable retest rate of 0.5%. The assay cutoff was the 0 value plus 6 SD of the negative samples. Thus a lower cutoff may be applied at the cost of a higher retest rate. Samples with a result >5 units/mL were tested in the ISAGA. Approximately 0.3% of the samples were tested in the ISAGA. Samples that tested positive in the /H9262/H9262-capture assay and the FEIA did not generally coincide with the samples that were positive in the AutoDELFIA assay. Approximately 50% of samples that were positive in both the FEIA and the AutoDELFIA assay were positive in the filter-paper ISAGA. If a sample...
was positive in the ISAGA, serum was requested from the mother and child, and the diagnosis was established by conventional serology (16). Almost all samples positive in the filter-paper ISAGA were confirmed on serum. Twenty-four newborns were finally diagnosed with CT. The sample results ranged from 6 to 669 units/mL with a mean of 133 units/mL and a median of 201 units/mL in the TOXO IgM+ A assay. All 24 samples were above cutoffs in the compared assays.

Samples with a result above the cutoff of 5 units/mL in the TOXO IgM+ A assay were tested to determine whether the response was of IgM or IgA type. These results showed that false-positive samples had an IgM response, whereas no false positives were IgA-positive. No samples were positive solely on their IgA-positive. Of the samples found truly toxoplasmosis-positive, 81% showed a positive IgA response.

**Discussion**

This study demonstrates that it is possible to use the AutoDELFIA system for detection of T. gondii-specific IgM and IgA antibodies and use it for neonatal screening. The great advantage of this method is that it can be run alongside an already established neonatal screening assay (AutoDELFIA neoTSH and/or neo17-hydroxyprogesterone; PLS-Wallac) with the same samples, automated instruments, software, and organization. The technical performance was validated by comparing the automated assay to an in-house μ-capture immunoassay, extensively characterized by clinical studies (1, 10), and the FEIA (15).

Direct labeling of the T. gondii antigen directly reduces the number of steps in this method compared with the μ-capture immunoassay. The antigen was SAG1, which was confirmed by immunoblotting with the SAG1-specific monoclonal antibody S13.

When the antigen is labeled with the Eu$^{3+}$ chelate of N$^1$-p-isothiocyanatobenzyl-diethylenetriamine-N$^1$,N$^2$,N$^3$-tetraacetic acid, the free amino groups of the protein are covalently bound to the isothiocyanato group, whereas the diethylenetriamine-N$^1$,N$^2$,N$^3$-tetraacetic acid forms a stable complex with Eu$^{3+}$ (14). The covalently bound group will add to the molecular mass of the molecule. It probably also increases the hydrophobicity of the antigen, causing it to aggregate. This may explain why the antigen performs so well in the assay with IgM, and the labeling procedure is optimized for this effect.

SDS destroys the hydrophobic bonds; therefore, the bands that appear in the Western blot were separate SAG1 molecules. The three bands that were stained by S13 and Toxoplasma-positive serum correspond to SAG1 labeled with 0, 3, and 6 Eu$^{3+}$ chelate molecules (0.667 kDa), respectively. The difference in labeling is probably attributable to differences in accessibility caused by the aggregation.

We found no increase when we combined anti-IgM and -IgA detection. The limited number of cases screened may explain this. Wallon et al. (8) identified 5 newborns with Toxoplasma-specific IgA but not IgM in a group of 89 Toxoplasma-infected newborns. In their study, Faure et al. (9) found no increased sensitivity when they used both IgA and IgM, and the number of true-positive cases was 18.

In conclusion, we have developed a clinically useful, automated TRIFMA for detection of Toxoplasma-specific IgM and IgA antibodies in filter-paper blood spots from neonates. The assay could be directly compared with an in-house μ-capture immunoassay (1) that showed a sen-
sitivity of 72%. Simultaneous anti-IgA detection did not increase the sensitivity.

We thank Lis Vestergaard-Hansen, Sanne Hovmand, Mona Rimestad, Lis Wasman, and Rehab Mohamed Maghrabi for excellent technical assistance.

References


