

Use of Recombinant Antigens for Early Postnatal Diagnosis of Congenital Toxoplasmosis

Wilma Buffolano,^{1†} Elisa Beghetto,^{2†} Mariassunta Del Pezzo,¹ Andrea Spadoni,²
Manlio Di Cristina,² Eskild Petersen,^{3,4} and Nicola Gargano^{2*}

Department of Pediatrics, Federico II University, Naples, Italy¹; Kenton Laboratories, Rome, Italy²; Department of Infectious Diseases, Aarhus University Hospital, Skejby, Denmark³; and Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden⁴

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The main objective of this work was to improve the early serologic diagnosis of toxoplasmosis in children at risk of congenital infection by using recombinant antigens. Serum samples from 104 infants born to mothers with primary *Toxoplasma gondii* infection acquired during pregnancy, of which 35 were congenitally infected and 22 had clinical silent toxoplasmosis at birth, were included. Immunoglobulin M (IgM), IgG, and IgG subtype antibodies against epitopes carried by fragments of *T. gondii* MIC2, MIC3, MIC4, M2AP, AMA1, and SAG1 gene products were measured by performing parallel enzyme immunoassays (Rec-ELISAs). Recombinant antigens preferentially reacted with IgG antibodies from infected infants compared to uninfected subjects ($P < 0.0001$), indicating that sera from infected children recognized a more diverse repertoire of antigens than sera transferred over the placenta from the mothers. Using two serial samples collected within 3 months of life, it was possible to demonstrate a neosynthesis of specific anti-MIC2 and anti-SAG1 immunoglobulin G, mainly of the IgG2 subtype, in 13 out of 20 infants with congenital toxoplasmosis. IgM antibodies in 97% of infected infants reacted with at least one of the recombinant antigens, confirming the diagnosis of congenital infection as soon as 2 months after birth ($P < 0.0001$). The use of recombinant antigens is effective in distinguishing *T. gondii*-infected from uninfected infants and shows that assays based on recombinant antigens improve the diagnosis of newborns with congenital toxoplasmosis.

Primary infection with *Toxoplasma gondii* acquired during pregnancy can be transmitted to the fetus and may cause miscarriage, neonatal malformations, or reduced eyesight (15, 37, 43). Toxoplasmosis in gestation represents a challenge for the clinician due to its subclinical course in the majority of pregnant women and to the unpredictable long-term outcome of congenital infection. Transplacental transmission occurs in 10 to 80% of maternal infections, depending on gestational age of the fetus (10, 11). The clinical severity for the fetus decreases and the transmission rate increases as gestational age at the time of maternal infection progresses (10, 21).

At birth, up to 90% of congenitally infected infants are asymptomatic but are at risk of developing retinochoroiditis during the first year of life or in early adulthood. Evidence from cohort studies shows that 15% to 80% of children with prenatal toxoplasmosis develop ocular disease (19, 25–27). Treatment should be started soon after birth, which requires rapid diagnosis (11, 19, 32, 42).

Detection of fetal *T. gondii* infection before birth can be established using PCR assays or isolating parasites by mouse inoculation from amniotic liquid samples. However, diagnosis of congenital toxoplasmosis during pregnancy by PCR and/or mouse inoculation usually identifies no more than 60 to 70% of the infected fetuses (13, 24, 38, 39, 41). Thus, exploring the antibody response to *T. gondii* in the newborn child seems an

obvious approach to improve the neonatal diagnosis of congenital toxoplasmosis.

Detection of *Toxoplasma*-specific immunoglobulin M (IgM) and IgA antibodies after birth confirms the diagnosis of congenital toxoplasmosis. However, in 30% to 60% of infected children the *Toxoplasma*-specific IgM and/or IgA antibody responses are absent or undetectable using standard serological assays, using lysed whole-cell *T. gondii* antigen (6, 7, 12, 37). Only the persistence or increase of IgG antibodies within the first 12 months of life can confirm congenital infection in the absence of clinical signs. To overcome this extended time lag between diagnosis and initiation of therapy, several additional tests based on comparison of the mother's immunological profile to that of her child have been developed (8, 17, 30, 34–36, 38).

The aim of this study was to improve the early serologic diagnosis of toxoplasmosis in children at risk of congenital infection by using recombinant *Toxoplasma* antigens. Using sera from infants born to mothers with primary toxoplasmosis acquired during pregnancy, we found that recombinant antigens containing regions of the *T. gondii* MIC2, MIC3, MIC4, AMA1, M2AP, and SAG1 gene products in enzyme immunoassays improve early diagnosis of congenital toxoplasmosis in newborns.

MATERIALS AND METHODS

Patients. One hundred four infants born to mothers with primary toxoplasmosis in pregnancy and referred for postnatal follow-up at the Center for Perinatal Infection of Campania Region, Italy, were included in the study. Maternal diagnosis of primary *T. gondii* infection was based on seroconversion during gestation. All of the women were offered tests for the antenatal diagnosis of congenital toxoplasmosis. When informed consent was granted, amniotic fluid

* Corresponding author. Mailing address: Kenton Laboratories, Via Pontina km 30.400, 00040 Rome, Italy. Phone: 39-06-91611494. Fax: 39-06-91629012. E-mail: gargano@kenton.it.

† These authors contributed equally to this work.

was drawn and then analyzed by PCR for the presence of *T. gondii*-specific nucleic acids (18). Prenatal diagnosis on amniotic fluid was attempted for 24 of the 104 women. The remaining 100 women did not consent or seroconverted late in gestation.

Treatment. (i) Mothers. We treated 74 women with spiramycin (3 g/day) alone up to delivery. After 16 weeks of gestation, a 4-week regimen alternating the combination of pyrimethamine (50 mg/day for the first 3 days and then 25 mg/day) plus sulfadiazine (3 g/day) and folic acid supplementation (P/S therapy) with spiramycin (3 g/day) was instituted in 19 women and continued up to delivery as follows: (i) first maternal IgM-positive test after 20 weeks of gestation (10, 15, 16); (ii) positive PCR result on amniotic fluid (22); and (iii) ultrasonographic anomalies (14). In 11 women treatment was lacking since the diagnosis was made close to delivery or because of the patient's refusal.

(ii) Infants. Treatment of infants was started when specific anti-*Toxoplasma* IgM and/or IgA was detected in the infant serum or a lack of IgG decay was demonstrated in two consecutive samples taken more than 2 weeks apart during the diagnostic follow-up. The disease onset was considered severe, benign, or subclinical according to the criteria of Hohlfeld et al. (21). Treatment of cases of severe-onset disease consisted of 6 months of P/S combination (pyrimethamine, 2 mg/kg of body weight per day in the first 3 days, then 1 mg/kg alternating days; sulfadiazine, 100 mg/day) and folic acid supplementation (5 mg/day, alternating days) followed by 6 months of a regimen alternating 4 weeks of the P/S combination (same dosage as above) with 4 weeks of spiramycin (125 mg/day). Cases of benign or subclinical disease were treated for 12 months with the regimen alternating 4 weeks of P/S combination with 4 weeks of spiramycin.

Diagnosis of congenital toxoplasmosis in newborns. Postnatal diagnosis in infants was carried out by determining specific anti-*Toxoplasma* IgM and IgA in the first 3 months of age and IgG at birth and at 1, 2, 3, 6, 9, and 12 months of age. Congenital toxoplasmosis was proven on the basis of persistence of *Toxoplasma*-specific IgG beyond 12 months of age and was excluded by their disappearance. Brain ultrasonography, cranial computed axial tomography, and indirect funduscopy established the severity of clinical onset (21).

Analysis of *Toxoplasma*-specific IgG, IgM, and IgA antibodies and measurement of anti-*Toxoplasma* IgG titers (given as international units/ml) in infant sera was done by the whole-cell, lysed *Toxoplasma* antigen assays ELFA-IgG, ELFA-IgM, and ISAGA-IgM (bioMérieux, Marcy-l'Étoile, France) and enzyme-linked immunosorbent assay (ELISA)-IgA (Sanofi-Pasteur, Marnes La Coquette, France). Sera taken from 30 *Toxoplasma* IgG-negative women referred to the center for other infectious diseases (cytomegalovirus, hepatitis C virus, or rubella virus) were used as controls. All serum samples were analyzed in a blinded fashion.

Recombinant antigens. Seven *Toxoplasma gondii* antigens were used: MIC2a, MIC2b, MIC4, M2AP, AMA 1 (5), MIC3, and SAG1 (3). Recombinant antigens were expressed in bacterial cells as fusion proteins with glutathione *S*-transferase (GST) and purified by affinity chromatography as previously described (3). Briefly, recombinant *Escherichia coli* cells were induced with IPTG (isopropylthiogalactopyranoside), centrifuged and suspended in STE buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl) containing 100 µg/ml of lysozyme and protease inhibitor cocktail (Boehringer, Germany). The mixture was sonicated at 4°C and Triton-X 100 was added to a final concentration of 1%. After centrifugation at 10,000 × *g* for 30 min at 4°C, the supernatant was recovered and incubated with glutathione-Sepharose (Amersham Pharmacia Biotech, Sweden). Fusion proteins were eluted by following the manufacturer's instructions and stored at -20°C.

Recombinant protein enzyme immunoassays. (i) IgG Rec-ELISA. Maxisorb plates (Nunc, Denmark) were coated overnight with recombinant proteins at a concentration of 5 µg/ml in coating buffer (50 mM NaHCO₃, pH 9.6). Plates were blocked for 1 h at 37°C with blocking buffer (5% nonfat dry milk, 0.05% Tween 20 in phosphate-buffered saline [PBS]) and then incubated for 1 h at 37°C with serum samples (diluted 1:200). After being washed with 0.05% Tween 20 in PBS, the plates were incubated with alkaline phosphatase-conjugated anti-human IgG antibodies (Sigma-Aldrich), diluted 1:7,500 in blocking buffer. After 30 min at 37°C the plates were washed and incubated with the chromogenic substrate *p*-nitrophenylphosphate (pNPP; Sigma-Aldrich) in developing solution (10% diethanolamine [pH 9.8], 0.5 mM MgCl₂, 0.05% Na₂S₂O₈). Results were recorded as the difference between the optical density (OD) at 405 and 620 nm using an automated ELISA reader (Multiskan Labsystems, Finland). For each serum, the assay was done in triplicate and the values given represent the mean of triplicate determinations. For every GST fusion product the cutoff value was determined as the mean plus two times the standard deviation (SD) of the absorbency readings obtained from the *Toxoplasma*-negative sera.

(ii) IgM Rec-ELISA. Maxisorb plates were coated with anti-human IgM antibodies (Sigma-Aldrich) at a concentration of 10 µg/ml in coating buffer. Plates

were blocked as above and subsequently incubated for 1 h at 37°C with serum samples (diluted 1:50). Plates were washed and then incubated for 3 h at room temperature with the GST fusion proteins at a concentration of 50 µg/ml. After being washed, the plates were incubated for 1 h at room temperature with anti-GST horseradish peroxidase-conjugated antibody (Sigma-Aldrich), diluted 1:5,000 in blocking solution. Finally, the enzymatic activity was revealed using the substrate tetramethylbenzidine (Sigma-Aldrich). Results were recorded as the difference between the absorbance at 450 and 620 nm. For each sample the assay was done in triplicate, and for every GST protein the cutoff value was determined as the mean plus 2 SD of the absorbency readings obtained with sera from uninfected infants.

(iii) IgG subtype Rec-ELISA. Maxisorb plates were coated with the recombinant proteins as described above and then blocked for 1 h at 37°C with 0.2% gelatin and 0.05% Tween 20 in PBS. Plates were incubated for 1 h at 37°C with sera (diluted 1:100). After being washed the plates were incubated for 1 h at 37°C with biotin-conjugated antibodies specific for human IgG1, IgG2, IgG3, and IgG4 (Sigma-Aldrich) diluted in blocking solution at 1:1,000 for IgG1 and IgG3 and at 1:5,000 for IgG2 and IgG4. Plates were washed and then incubated for 30 min at 37°C with avidin-alkaline phosphatase (Sigma Aldrich) diluted 1:10,000 in blocking solution. Finally, the enzymatic activity was revealed using the substrate pNPP as described above. For each serum sample, the assay was done in triplicate and average values were calculated. For each assay, the cutoff value was determined as the mean plus 2 SD of the absorbency readings obtained from the *Toxoplasma*-negative sera.

Statistical analysis. Proportions were compared by chi-square test with *P* < 0.05 considered statistically significant. The receiver operating characteristic (ROC) curves were calculated using the Excel-based statistical package Analyse-it (<http://www.analyse-it.com/>, accessed 25 May 2005).

RESULTS

In a retrospective study, sera from 104 infants of mothers with primary *Toxoplasma gondii* infection during pregnancy were analyzed. Thirty-five infants had congenital toxoplasmosis and 69 were uninfected, as demonstrated by the persistence or disappearance, respectively, of *Toxoplasma*-specific IgG antibodies after 12 months of age. Table 1 shows the characteristics of the infected-patient cohort. The gestational age at the time of maternal infection was the second trimester in 15 mothers and the third trimester in 20 mothers. Detection of fetal *T. gondii* infection by PCR from amniotic fluid was performed for only four women (mothers of patients T6, T18, T19, and T25) because of the patient's refusal or primary infection acquired late in gestation, giving a positive result in only one case (patient T6). All but six of the mothers were treated, and 17 women received the regimen alternating P/S therapy with spiramycin after 16 weeks of gestation up to delivery. Infant sera were tested before starting postnatal treatment in all but five of the patients. According to the criteria of Hohlfeld et al. (21), disease onset was considered severe in five infants, benign in eight, and subclinical in 22.

Seven antigenic regions of the *T. gondii* polypeptides MIC2a, MIC2b, MIC3, MIC4, M2AP, AMA1, and SAG1, which had been shown previously to contain B-cell epitopes (3, 5), were expressed as fusion products with the bacterial protein GST. Immunoreactivity of GST fusion proteins with IgG antibodies in sera from infected and uninfected infants was determined. Each of the antigens was coated separately onto individual microtiter wells and tested in the IgG Rec-ELISAs with sera from the 104 newborns. Thirty *Toxoplasma*-negative sera were also assayed as controls. Specific levels of anti-*Toxoplasma* IgG, determined by the whole-cell *Toxoplasma* immunoassay (VIDAS system from Biomerieux) (31), ranged from 26 to 2,549 IU/ml for sera from infected infants, from 9 to 427 IU/ml

TABLE 1. Characteristics of the infected infants

Patient no.	Maternal infection ^a	Prenatal treatment ^b	Onset ^c	Clinical manifestations ^d			Postnatal treatment ^e
				RC	EC	HC	
T1	II	Spir	B	-	+	-	-
T2	II	Spir	Sub	-	-	-	-
T3	III	Spir	Sub	-	-	-	-
T4	III	Spir	Sub	-	-	-	-
T5	III	P/S	B	+	-	-	-
T6	II	P/S	B	-	+	-	-
T7	II	P/S	Sub	-	-	-	-
T8	III	P/S	B	-	+	-	-
T9	III	P/S	Sub	-	-	-	-
T10	III	P/S	B	+	+	+	+
T11	III		Sub	-	-	-	-
T12	II	Spir	Sub	-	-	-	-
T13	III	P/S	Sub	-	-	-	-
T14	III	P/S	Sub	-	-	-	-
T15	III		B	-	+	-	-
T16	III		Sub	-	-	-	-
T17	III	Spir	Sub	-	-	-	-
T18	II	Spir	B	-	+	-	-
T19	II	P/S	Sub	-	-	-	-
T20	III		Sub	-	-	-	-
T21	III	P/S	S	+	+	-	-
T22	III	P/S	Sub	-	-	-	-
T23	II	Spir	Sub	-	-	-	-
T24	II	P/S	B	-	+	-	-
T25	II	P/S	S	+	+	+	+
T26	II	Spir	S	+	+	+	+
T27	III	Spir	Sub	-	-	-	-
T28	II	Spir	S	+	+	-	+
T29	III		Sub	-	-	-	-
T30	III	Spir	S	+	+	-	-
T31	III		Sub	-	-	-	-
T32	III	P/S	Sub	-	-	-	-
T33	III	P/S	Sub	-	-	-	-
T34	III	P/S	Sub	-	-	-	+
T35	III	P/S	Sub	-	-	-	-

^a Trimester of gestation.^b Spir, spiramycin; P/S, pyrimethamine-sulfadiazine in combination with spiramycin.^c Severity of clinical onset: S, severe; B, benign; Sub, subclinical.^d RC, retinochoroiditis; EC, endocranial calcifications; HC, hydrocephalus.^e Treatment of the infected infant at the time of serum collection.

for sera from uninfected subjects, and from 0 to 6 IU/ml for the negative controls.

As shown in Table 2, with the exception of MIC4 and AMA1, all of the fusion proteins reacted with more than 75% of the sera from the congenitally infected infants. One of the 30 *Toxoplasma*-negative sera did react with the GST-AMA1 antigen and none recognized the GST-MIC2a, GST-MIC2b,

GST-MIC3, GST-MIC4, GST-M2AP, and GST-SAG1 antigens. Interestingly, the number of reactive sera from infected versus uninfected infants, reflecting maternal antibodies, was significantly different ($P < 0.0001$). In particular, the recombinant GST-MIC3 reacted with IgG antibodies in 94% of sera from infected infants, while it failed to react with 71% of sera from uninfected subjects, indicating that sera from infected children recognized a more complex repertoire of antigens than sera from the mothers. Overall, IgG antibodies from 100% of the congenitally infected patients reacted with at least one recombinant antigen, while sera from 23 out of 69 uninfected subjects did not react with any recombinant product (data not shown).

Next, we examined the immunoreactivity of recombinant antigens with immunoglobulin M of infant sera. To this aim, a double-sandwich immunoassay was employed (IgM Rec-ELISA). The GST-MIC2a, GST-MIC2b, GST-MIC3, GST-M2AP, and GST-SAG1 fusion proteins were assayed with the 104 serum samples from infected and uninfected babies, and the results obtained with commercial assays employing either lysed whole-cell *T. gondii* antigen or fixed parasites (ELFA-IgM and ISAGA-IgM, respectively) were compared. As a control, the IgM reactivity against wild-type GST protein was assessed for each serum. The diagnostic criterion used to assign a positive IgM reactivity against single antigens was an $OD_{\text{GST-antigen}}$ greater than the cutoff and an $OD_{\text{GST-antigen}}$ greater than the OD_{GST} .

As shown in Table 3, the number of IgM-reactive sera ranged from 66% to 31% using the GST-SAG1 and GST-MIC3 antigens, respectively. Among uninfected infants, IgM antibodies in 1.4% of newborns reacted with the GST-MIC2b and GST-M2AP antigens, those in 4.3% of infants reacted with GST-SAG1, and none recognized the GST-MIC2a and GST-MIC3 antigens, indicating the occurrence of few unspecific reactions. In Table 4 are summarized the results of the IgM Rec-ELISAs with individual sera from infected infants. Overall, IgM antibodies in 34 out of 35 sera from the congenitally infected patients reacted with at least one recombinant antigen. Notably, all sera from infected infants with silent toxoplasmosis (subclinical onset, see Table 1) were reactive using the IgM Rec-ELISAs. In contrast, only four of them (patients T3, T11, T29, and T32) had positive results when the whole-cell assays were employed.

The clinical usefulness of recombinant antigens in the IgM-capture assay was investigated further by analyzing in detail the diagnostic performance of single recombinant antigen assays and combinations of assays. The ROC curves for the individual

TABLE 2. *Toxoplasma*-specific IgG reactivity of 104 serum samples from 104 infants of mothers with primary *T. gondii* infection in pregnancy

Group ^a	No. of samples	Mean IgG level ^b (IU/ml)	No. (%) [P] ^c of samples reactive in IgG Rec-ELISA against:						
			MIC2a	MIC2b	MIC3	MIC4	M2AP	AMA1	SAG1
Infected	35	338	34 (97)	29 (83)	33 (94)	17 (49)	31 (89)	20 (57)	24 ^d (75)
Uninfected	69	61	34 (49) [<0.0001]	22 (32) [<0.0001]	20 (29) [<0.0001]	2 (3) [<0.0001]	30 (43) [<0.0001]	10 (15) [<0.0001]	23 (33) [<0.0001]
Controls	30		0	0	0	0	0	1	0

^a Serum samples collected from: A, newborns with congenital toxoplasmosis; B, uninfected infants; C, *Toxoplasma*-seronegative controls.^b *Toxoplasma*-specific IgG levels, expressed as International Units (IU).^c Chi-square test comparing uninfected and infected newborns.^d Only 32 samples tested against SAG1.

TABLE 3. *Toxoplasma*-specific IgM reactivity of 104 serum samples from 104 infants of mothers with primary *T. gondii* infection in pregnancy^a

Group	No.	No. (%) of reactive sera [P]						
		ELFA IgM	ISAGA IgM	IgM Rec-ELISA				
				MIC2a	MIC2b	MIC3	M2AP	SAG1
Infected	35	9 (26)	9 (26)	21 (60)	18 (51)	11 (31)	17 (49)	23 (66)
Uninfected	69	0	0	0 [<0.0001]	1 (1.4) [<0.0001]	0 [<0.0001]	1 (1.4) [<0.0001]	3 (4.3) [<0.0001]

^a Values indicate the number of reactive sera using commercial assays (ELFA and ISAGA) or recombinant *T. gondii* antigens.

antigens clearly show that the IgM Rec-ELISA using the recombinant SAG1 antigen has the best performance, followed by the assays employing MIC2a, MIC2b, and MIC3 antigens, whereas the whole lysed *Toxoplasma* antigen (ELFA assay) displays the worst performance (Fig. 1). Table 5 shows the statistics for the single IgM Rec-ELISAs compared to the results obtained with the most relevant combinations of recombinant antigen immunoassays. The best outcome, in terms of sensitivity, agreement, and negative predictive value, was

found when results from the MIC2a, MIC2b, and SAG1 IgM Rec-ELISAs were combined. With regard to specificity and positive predictive value, the best outcome was found when the results from the MIC2a and MIC3 assays were combined.

Table 6 shows the performance characteristics of the commercial assays (ELFA-IgM, ELISA-IgA, and ISAGA-IgM), employed as individual tests or in combination, compared to the results obtained with the array of recombinant antigen IgM assays (five-antigen Rec-ELISAs). The best outcome, in terms

TABLE 4. Immunoreactivity of recombinant antigens with IgM antibodies in serum samples from infants with congenital toxoplasmosis^a

Patient no.	Time after birth (wk)	IgG level (IU/ml)	ELISA IgA result	ISAGA IgM result	ELFA IgM cutoff ^b	Rec-ELISA IgM cutoff ^c				
						MIC2a	MIC2b	MIC3	M2AP	SAG1
T1	1	1,192	Pos.	Pos.	1.22	1.13	0.78	0.69	1.03	0.88
T2	1	300	Neg.	Neg.	0.09	0.59	0.74	0.73	0.73	1.46
T3	1	217	Pos.	Pos.	7.00	1.13	0.95	1.29	0.80	0.91
T4	1	153	Neg.	Neg.	0.08	1.42	1.28	1.21	1.69	1.60
T5	1	988	Neg.	Neg.	0.03	0.86	0.67	0.95	0.45	1.44
T6	2	39	Neg.	Neg.	0.06	0.82	0.76	0.69	0.50	1.17
T7	2	673	Neg.	Neg.	0.11	1.05	1.30	1.01	1.45	1.54
T8	2	206	Neg.	Neg.	0.05	0.25	0.61	0.92	0.73	0.94
T9	2	124	Neg.	Neg.	0.13	1.62	1.57	1.67	1.76	1.78
T10	2	226	Pos.	Pos.	0.91	1.39	1.01	0.76	0.65	0.87
T11	2	157	Pos.	Pos.	7.50	0.61	0.63	0.49	0.85	1.31
T12	3	148	Neg.	Neg.	0.07	1.21	1.12	1.04	0.79	1.57
T13	3	57	Neg.	Neg.	0.05	0.88	0.69	0.91	0.47	1.54
T14	3	73	Neg.	Neg.	0.08	1.18	1.08	0.97	1.22	1.39
T15	3	161	Pos.	Pos.	4.02	1.18	1.44	1.22	0.62	1.31
T16	3	191	Neg.	Neg.	0.18	1.00	1.92	0.77	1.17	1.00
T17	3	96	Neg.	Neg.	0.08	0.96	0.77	0.71	0.70	1.32
T18	4	195	Neg.	Neg.	0.11	0.63	1.01	1.38	0.77	0.97
T19	4	103	Neg.	Neg.	0.13	1.35	1.17	1.18	1.09	1.52
T20	4	153	Neg.	Neg.	0.08	1.32	1.22	1.04	1.67	1.52
T21	4	578	Pos.	Pos.	5.95	0.96	0.88	0.93	0.95	1.45
T22	4	218	Neg.	Neg.	0.04	1.28	0.84	1.29	1.26	0.84
T23	5	51	Neg.	Neg.	0.09	0.69	1.09	0.71	1.11	0.98
T24	5	47	Neg.	Neg.	0.11	0.62	0.80	0.73	0.76	1.18
T25	5	1,147	Neg.	Neg.	0.07	0.78	0.96	1.67	0.59	1.26
T26	5	816	Pos.	Neg.	0.04	1.21	1.22	0.75	1.05	1.00
T27	5	26	Neg.	Neg.	0.11	0.98	0.89	0.77	1.13	0.94
T28	6	2,549	Neg.	Pos.	0.88	1.63	1.09	1.91	0.89	0.83
T29	6	292	Pos.	Pos.	5.77	1.59	1.41	1.94	1.42	1.52
T30	6	28	Neg.	Neg.	0.11	1.38	0.62	2.75	0.58	1.78
T31	7	90	Neg.	Neg.	0.04	1.25	1.01	1.00	1.86	1.31
T32	7	260	Neg.	Pos.	2.23	1.57	1.18	0.87	1.14	1.23
T33	8	145	Neg.	Neg.	0.31	1.18	1.04	0.80	0.88	1.31
T34	8	59	Neg.	Neg.	0.07	1.56	0.93	1.24	1.18	0.99
T35	8	67	Neg.	Neg.	0.12	1.30	1.11	1.28	1.73	1.63

^a Serum samples (T1 to T35) were analyzed using IgM Rec-ELISAs with individual GST fusion products or commercial assays employing whole-cell *Toxoplasma* antigen (ELISA IgA, ELFA IgM, and ISAGA IgM).

^b Cutoff value for the commercial ELFA-IgM assay was 0.65, as indicated by the manufacturer (bioMerieux, France). Bold type, values $>$ cutoff.

^c The numeric values were calculated as the ratio of the test OD to the cutoff of the corresponding antigen. Cutoff values for GST-MIC2a, GST-MIC2b, GST-MIC3, GST-M2AP, and GST-SAG1 were 0.40, 0.45, 0.35, 0.64, and 0.90, respectively. Bold type, $OD_{GST-antigen} >$ cutoff and $OD_{GST-antigen} >$ OD_{GST} .

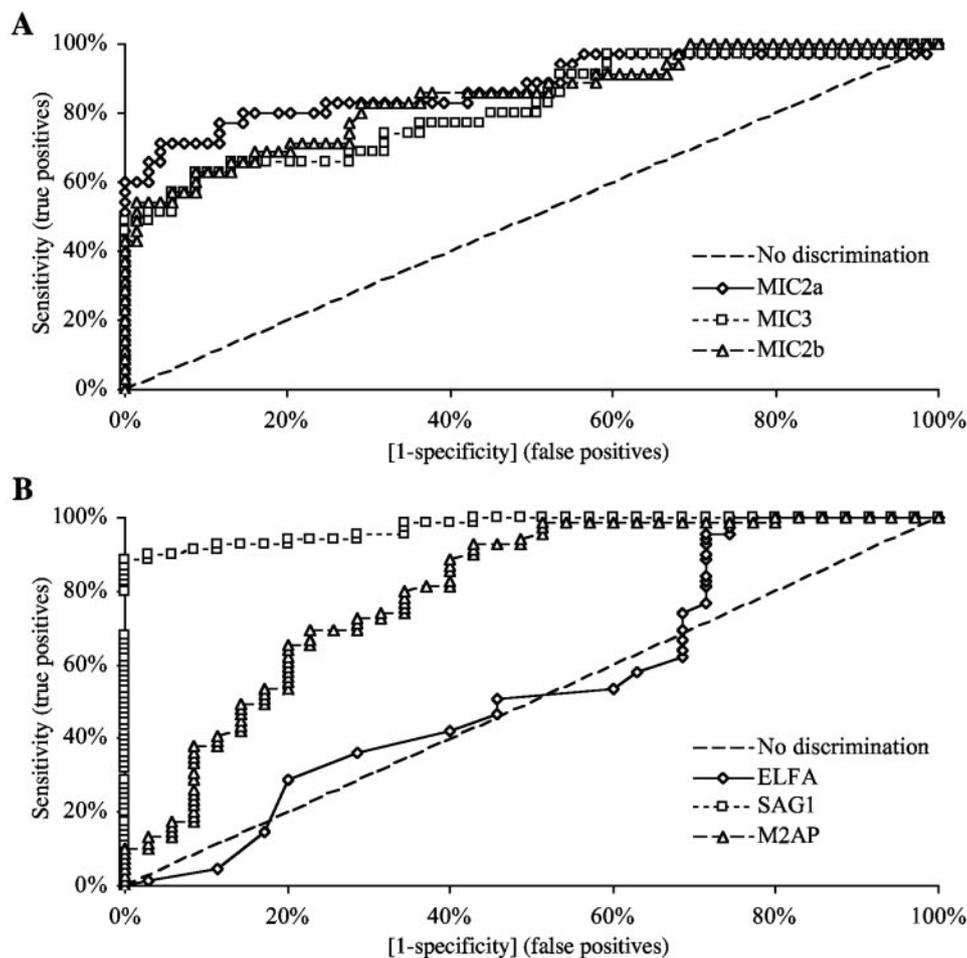


FIG. 1. Statistical analysis of the IgM capture immunoassays. ROC curves for the IgM capture immunoassays using the five recombinant antigens MIC2a, MIC2b, and MIC3 (panel A) and M2AP, SAG1, and the whole lysed *Toxoplasma* antigen (ELFA) (panel B).

of sensitivity, agreement, and negative predictive value, was found with results from the recombinant antigen IgM tests compared to the results obtained with commercial assays used alone or in any combination. With regard to specificity and positive predictive value, the best performance was found when the results from the commercial assays were combined.

We finally investigated the presence in the infant sera of specific anti-*Toxoplasma* IgG subtypes, which were unequiv-

ocally synthesized by newborns with congenital infection. The immunoreactivity of the GST-MIC2a and GST-SAG1 fusion proteins against IgG1, IgG2, IgG3, and IgG4 subtypes was assessed with two consecutive samples collected from each subject (the interval between sample collection was 2 to 12 weeks). We analyzed 40 serum samples from 20 infected infants, and 20 *Toxoplasma*-negative sera were also assayed as a control.

TABLE 5. Diagnostic performance of the IgM-capture assays based on recombinant antigens^a

IgM Rec-ELISA antigen(s)	Sensitivity (%)	Specificity (%)	Agreement (%)	PPV (%)	NPV (%)
MIC2a	60	100	86.5	100	83.1
MIC2b	51.4	98.6	82.7	94.7	80
MIC3	31.4	100	76.9	100	74.2
M2AP	48.6	98.6	81.7	94.4	79.1
SAG1	65.7	95.8	85.6	88.5	84.6
MIC2a + SAG1	85.7	95.8	92.3	90.9	93
MIC2a + MIC3	65.7	100	88.5	100	85.2
MIC2a + MIC2b + SAG1	94.3	94.5	94.2	89.2	97
MIC2a + MIC3 + SAG1	88.6	95.8	93.3	91.2	94.3
MIC2a + MIC2b + MIC3	71.4	98.6	89.4	96.2	87.2

^a PPV, positive predictive value; NPV, negative predictive value.

TABLE 6. Diagnostic performance of combinations of antibody-based immunoassays^a

Diagnostic test(s)	Sensitivity (%)	Specificity (%)	Agreement (%)	PPV (%)	NPV (%)
ELFA-IgM	25.7	100	75	100	72.6
ISAGA-IgM	25.7	100	75	100	72.6
ELISA-IgA	22.9	100	74	100	71.9
Rec-ELISA-IgM (5 antigens)	97.1	93.2	94.2	87.2	98.5
ELFA-IgM + ISAGA-IgM	25.7	100	75	100	72.6
ELFA-IgM + ELISA-IgA	28.6	100	76	100	73.4
ISAGA-IgM + ELISA-IgA	28.6	100	76	100	73.4
ELFA-IgM + Rec-ELISA-IgM	97.1	93.2	94.2	87.2	98.5
ISAGA-IgM + Rec-ELISA-IgM	97.1	93.2	94.2	87.2	98.5
ELISA-IgA + Rec-ELISA-IgM	97.1	93.2	94.2	87.2	98.5

^a PPV, positive predictive value; NPV, negative predictive value.

Table 7 shows the results obtained with the IgG subtype analysis. Overall, 13 out of 20 infected infants displayed an increase of specific IgG subtypes reactivity against the recombinant antigens between the first and 13th weeks after birth. Among these subjects, 12 (92%), 7 (54%), and 5 (38%) infected infants displayed an increase in specific IgG2, IgG3, and IgG1 reactivity, respectively, and only one (8%) displayed an increase in IgG4 reactivity against the recombinant *T. gondii* antigens. None of the IgG subclasses in sera from the negative controls reacted with the GST-MIC2a and GST-SAG1 antigens (data not shown).

DISCUSSION

To provide proper clinical care for newborns, it is important to have a sensible and accurate method for diagnosis to distinguish, early after birth, congenitally infected infants from uninfected babies born to mothers who acquired primary *Toxoplasma gondii* infection during gestation.

Because *Toxoplasma* infection crosses the placenta in only ~20% of maternal infections, screening out uninfected babies is an important goal to avoid unnecessary treatment. Also, identification of infected babies, whose infections are often subclinical at birth, is crucial to help avoid or minimize delayed sequelae, such as impaired mental development or ocular complications seen later in childhood or adolescence. As the wide spectrum of clinical manifestations in congenital toxoplasmosis overlaps those of other vertically transmittable diseases (e.g., cytomegalovirus, herpes simplex virus, rubella, and syphilis) (37) and the clinical expression of toxoplasmosis is absent in up to 85% of infected newborns (6, 15, 16, 22, 27), the diagnosis of congenital infection early after birth can only be made either indirectly by serological methods or directly by PCR from blood, other fluids, or tissue samples (for a review, see reference 29). Serological methods are generally preferred since the sensitivity of PCR results can be affected by the appropriateness of sample handling, shipping and storage conditions, and the technique used for PCR amplification and detection. Furthermore, parasitemia may be of short duration, further limiting the value of PCR of peripheral blood.

Serological diagnosis of congenital toxoplasmosis in the newborn is most commonly made by detecting *Toxoplasma*-specific IgM and/or IgA antibodies in infant serum. However, *Toxoplasma*-specific IgM and IgA antibodies are usually found in only up to 75% of infected babies (6, 7, 15, 16, 22, 27).

Most commercial assays for detecting specific anti-*Toxoplasma* immunoglobulins use the whole *T. gondii* soluble extract as the antigen. Consequently, the immunoassay will take into account the relative concentrations of the specific anti-*Toxoplasma* antibodies in serum and the corresponding antigens in the parasite extract. Our hypothesis was that using a combination of recombinant antigens individually challenged with antibodies from infected individuals might enhance the sensitivity of an antibody-based immunoassay. Moreover, a major advantage of using recombinant antigens is the higher reproducibility of assays, which is the first step toward a real standardization of toxoplasmosis serodiagnosis.

Several previous studies have found that recombinant antigens improve the serological diagnosis of *T. gondii* infections in adults with acquired infection (1, 4, 20, 23, 28, 33, 40). However, no studies so far have described the use of recombinant antigens for the diagnosis of congenital toxoplasmosis in newborns.

Immunodominant regions of *T. gondii* proteins have previously been identified (2–5, 9). The immunoreactivity of seven distinct fragments of *T. gondii*, the MIC2, MIC3, MIC4, M2AP, AMA1, and SAG1 proteins, expressed as GST fusion products, was assessed with sera from infants with congenital *T. gondii* infection. IgG antibodies from all infected infants reacted with at least one of the antigen fragments, emphasizing the immunogenicity of these proteins in humans. The recombinant MIC3 and MIC4 reacted prevalently with IgG antibodies from the congenitally infected infants versus uninfected babies, even though all of the newborn sera contained maternal IgG antibodies. This suggests that specific anti-MIC3 and anti-MIC4 IgG antibodies may indicate actively replicating *T. gondii*, whereas they disappear in subjects with latent *T. gondii* infection.

To discriminate between IgG antibodies transferred during gestation from the mother to her fetus from those newly synthesized by infants after birth, we analyzed the immunoreactivity of recombinant antigens against the distinct subtypes of immunoglobulins G in sera from newborns with congenital toxoplasmosis. In 13 of 20 infected newborns it was possible to highlight the presence of infant-specific IgG antibodies. The synthesis of *Toxoplasma*-specific IgG by infants appears to involve mainly the IgG2 and IgG3 subtypes, while the level of IgG1 subtype generally decreases soon after birth. This suggests that IgG1 is prevalently transferred from the mother

TABLE 7. Immunoreactivity of recombinant antigens with IgG subtype antibodies in serum samples from infants with congenital *T. gondii* infection^a

Patient no.	IgG level (IU/ml)	Time after birth (wk)	MIC2a Rec-ELISA cutoff				SAG1 Rec-ELISA cutoff			
			IgG1	IgG2	IgG3	IgG4	IgG1	IgG2	IgG3	IgG4
T1	905	7	23.5	1.0	2.5	1.0	7.7	1.2	0.5	0.7
	220	13	<i>10.4</i>	1.0	<i>1.3</i>	0.8	<i>4.9</i>	1.5	0.5	0.7
T2	300	1	<i>4.3</i>	<i>1.2</i>	<i>1.1</i>	0.8	<i>2.9</i>	<i>4.3</i>	<i>1.6</i>	0.9
	275	4	2.9	0.7	0.7	0.7	<i>1.8</i>	<i>1.5</i>	0.9	0.6
T4	153	1	3.6	2.3	1.5	1.0	1.1	0.7	0.4	0.5
	87	5	<i>2.1</i>	2.6	1.4	1.0	1.3	2.4	1.2	0.8
T5	277	6	2.3	0.8	0.7	0.8	1.0	1.3	0.7	0.7
	146	10	<i>1.5</i>	0.8	0.6	0.9	0.9	1.5	0.6	0.6
T7	240	7	2.6	2.0	0.8	0.9	1.4	0.7	0.8	0.6
	190	11	2.6	<i>1.5</i>	1.2	0.8	2.0	1.3	1.0	0.5
T8	206	2	2.3	0.9	0.8	0.8	<i>2.0</i>	1.6	0.6	0.9
	64	8	<i>1.4</i>	0.8	0.7	0.9	<i>1.2</i>	2.8	0.6	1.0
T9	124	2	4.0	2.5	1.3	1.0	2.7	2.6	<i>1.8</i>	<i>1.5</i>
	73	7	2.8	4.1	1.7	1.2	<i>1.4</i>	4.4	<i>1.3</i>	1.0
T10	226	2	3.9	1.2	1.1	1.0	2.4	1.1	0.9	0.5
	216	4	2.3	1.7	1.8	0.7	1.9	0.7	0.8	0.5
T11	157	2	0.9	0.8	<i>1.2</i>	0.7	0.9	1.1	1.0	0.8
	140	7	3.0	0.9	0.9	0.7	1.2	1.3	0.7	0.6
T12	148	3	2.7	1.3	1.1	0.7	<i>1.1</i>	1.5	1.2	0.6
	136	6	<i>2.0</i>	1.9	1.2	0.9	<i>1.1</i>	2.0	1.5	0.6
T13	57	3	0.9	0.9	<i>1.2</i>	0.8	0.8	<i>1.3</i>	<i>1.1</i>	0.6
	39	8	0.8	1.2	0.8	0.8	0.7	<i>1.2</i>	0.6	0.6
T14	73	3	<i>1.3</i>	1.0	0.9	0.7	0.7	0.6	0.3	0.6
	43	9	0.9	0.9	0.8	0.7	0.7	0.5	0.4	0.5
T20	153	4	2.6	0.8	0.8	0.5	<i>1.1</i>	0.7	0.6	0.5
	132	8	<i>1.9</i>	0.9	0.8	0.6	0.9	0.7	0.5	0.6
T22	218	4	2.0	0.9	0.8	0.7	<i>1.9</i>	<i>1.3</i>	0.9	0.8
	221	6	<i>1.6</i>	0.9	0.7	0.7	<i>1.3</i>	<i>1.1</i>	0.8	0.8
T24	47	5	0.9	<i>1.1</i>	0.6	0.8	<i>1.3</i>	2.0	0.5	0.7
	30	9	0.9	0.9	0.6	0.8	0.8	2.3	0.5	0.6
T25	1,147	5	<i>11.8</i>	<i>1.2</i>	2.7	0.6	2.3	<i>1.2</i>	0.7	0.7
		17	<i>1.2</i>	0.9	1.0	0.8	0.8	0.9	0.7	0.5
T29	292	6	25.2	<i>1.3</i>	5.8	0.7	2.9	0.8	<i>1.1</i>	0.6
	300	10	<i>14.6</i>	<i>1.1</i>	3.8	0.5	2.8	0.7	0.8	0.5
T30	39	3	2.2	0.9	1.3	0.7	0.8	1.0	0.4	0.5
	28	6	9.7	1.0	8.3	0.7	0.8	0.9	0.3	0.5
T34	59	8	3.3	0.7	0.9	0.9	<i>1.1</i>	1.1	0.4	0.8
	176	13	6.9	0.9	3.1	1.0	1.0	1.6	0.6	0.6
T35	91	4	<i>1.3</i>	0.9	0.8	0.5	<i>1.3</i>	<i>1.7</i>	1.0	0.4
	67	8	0.9	0.8	0.4	0.4	1.0	0.9	0.5	0.5

^a Serial serum samples from infants with congenital toxoplasmosis were analyzed using IgG subtype Rec-ELISAs with GST fusion products. The values were calculated as the ratio of the test OD to the cutoff of the corresponding antigen. Italic type, OD > cutoff; bold type, serum sample pairs showing increasing IgG subtype reactivity with time.

during gestation, while synthesis of the IgG2 and IgG3 subtypes is mainly directed by infants in response to congenital *T. gondii* infection.

The recombinant antigens were assayed with IgM antibodies in sera from the patient cohorts by using a double-sandwich immunoassay, and the results of the IgM Rec-ELISAs were compared with those obtained with whole-cell *Toxoplasma* antigen. Overall, 97% of infants (34/35) with congenital toxoplasmosis were found to be positive using the IgM Rec-ELISA analysis, whereas only 29% of infected individuals (10/35) were detected by the whole-cell immunoassays (combination of ISAGA-IgM or ELFA-IgM plus ELISA-IgA; see Table 6).

The IgM assays with recombinant antigens generally displayed a good performance in terms of sensitivity and specificity. Only with the SAG1 antigen were a few false-positive results (3 out of 69 negative controls) observed, probably due to unspecific reactions of IgM antibodies from the sera of the uninfected infants. However, the false-positive results could be avoided by increasing the cutoff value of the SAG1 assay (e.g., using 3 SDs), although this resulted in missing two cases of infected infants (T6 and T24) (data not shown). Therefore, a lower cutoff value was employed despite reduced assay specificity.

It is noteworthy that 22 of the infected infants had a subclinical form of congenital toxoplasmosis, thus representing suspected cases of *T. gondii* infection, which were clinically undiagnosed at birth. Our results indicate that by employing an IgM immunoassay based on recombinant antigens, it was possible to correctly confirm the congenital infection in all of these subjects as soon as 8 weeks after birth, thus potentially allowing prompt treatment for patients with asymptomatic toxoplasmosis in early infancy.

In conclusion, this study demonstrates that the use of recombinant antigens is effective in distinguishing *T. gondii*-infected from uninfected infants and could provide the basis of valuable serologic tests for improving early diagnosis of newborns with congenital toxoplasmosis.

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