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Utility of Immunoblotting for Early Diagnosis of Toxoplasmosis Seroconversion in Pregnant Women▽

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Congenital transmission of *Toxoplasma gondii* occurs mainly when a mother acquires the infection for the first time during pregnancy. It was recently shown that although early treatment of the primary infection during pregnancy has little or no impact on the fetomaternal transmission rate, it does reduce the incidence of sequelae in infected infants. Seroconversion is defined by the appearance of IgG. Commercial reagents continue to vary considerably in detecting low concentrations of antibodies, as during early seroconversion. We compared two routinely used immunoassays (IA) (Platelia and Elecsys Toxo IgG) and an indirect immunofluorescence assay (IIF) with a qualitative test based on immunoblot analysis (Toxo II IgG) (IB) to assess their abilities to diagnose seroconversion at its earliest stages. This prospective study was carried out between January and November 2010. It included 39 pregnant women with monthly follow-up who seroconverted during pregnancy. On first sera that were IgM positive but IgG negative (or equivocal) as detected by IA, IB diagnosed seroconversion twice as often as IIF (26/39 [66.7%] versus 13/39 [33.3%]; $P < 0.001$; χ^2 test). Serum samples were retaken 2 to 5 weeks later for the other 13 cases (IgG negative by IB on first serum). Seroconversion was demonstrated as follows: IB for 5 cases where IA remained negative or equivocal, IB and IIF for 5 cases where IA remained negative or equivocal, IA for 2 cases, and no method for 1 case (a third sample was necessary). In summary, IB permitted toxoplasmosis seroconversion diagnosis before other means in 92.3% of cases (36/39) and thus earlier therapeutic intervention.

Congenital transmission of *Toxoplasma gondii* occurs mainly when a mother acquires the infection for the first time during pregnancy. Clinical manifestations of congenital toxoplasmosis at birth vary according to the stage of pregnancy at the time of infection from severe, if contamination occurs early during pregnancy, to asymptomatic in end-of-pregnancy contamination (2, 4). It was recently shown that although early treatment of the primary infection during pregnancy has little or no impact on the fetomaternal transmission rate, it does reduce the incidence of sequelae in infected infants (7). In France, approximately 2,500 cases of primary *Toxoplasma* infection are observed in pregnant women every year, with around 400 to 600 cases of congenital toxoplasmosis. Of these, 175 result in sequelae (French Food Safety Agency [AFSSA] data from 2006 [1]). The diagnosis of acute toxoplasmosis during pregnancy is difficult because it is usually subclinical or associated with nonspecific symptoms (13). Therefore, French legislation requires monthly serological monitoring of pregnant women (anti-*T. gondii* immunoglobulin M [IgM] and immunoglobulin G [IgG]) if their toxoplasmosis serology is negative before pregnancy. Usually, specific IgM appears 1 week after infection (10) and IgG 1 to 3 weeks after IgM (2). Toxoplasmosis seroconversion is defined by the appearance of IgG. The appearance of IgM alone is diagnostically awkward because it may be due to nascent toxoplasmosis seroconversion or a nonspecific IgM reaction (3, 14). Immunoenzymatic or chemiluminescence tests are the most frequently used serological diagnostic methods. However, commercial reagents continue to vary considerably in detecting low concentrations of antibodies. Indeed, our experience is that IgG concentrations detected with routine tests are often equivocal even though we routinely use a second confirmatory test. For all these reasons, sensitive and specific IgG detection methods are necessary to detect seroconversion as early as possible in pregnant women (17). Early diagnosis of toxoplasmosis infection in this population would enable fast and appropriate therapeutic intervention and decrease the incidence of sequelae in infected infants. The aim of this study was to compare routinely used tests (immunoenzymatic and chemiluminescence tests) and a classical method, the indirect immunofluorescence assay (IIF), with a qualitative test based on immunoblotting to assess their abilities to diagnose seroconversion in its earliest stages.

(Some of the data in this study were presented at the IVth International Congress on Congenital Toxoplasmosis [ICOCT], October 2010, Marseille, France.)

MATERIALS AND METHODS

Patients. This prospective study was carried out between January and November 2010. It included 39 pregnant women in whom monthly monitoring detected seroconversion during pregnancy. Samples came from several laboratories located throughout France. They referred to our laboratory samples for which Elecsys Toxo IgM determination was positive and Elecsys Toxo IgG determination was negative or equivocal.

Methods. All results obtained with Elecsys reagents were available. Each sample referred to our laboratory was subjected to 3 testing methods to detect IgG (immunoblot analysis [Toxo II IgG] [IB], IIF, and Platelia IgG) and 2 to detect IgM (Platelia IgM and Toxo-ISAgA IgM).

The Elecsys Toxo test is an electrochemiluminescence immunoassay (ECLIA)

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TABLE 1. Study of the first sera positive for IgM but negative or equivocal for IgG by routine methods

IB[a]	No. of sera with indicated result								
	IgG Platelia		IgG Elecsys				IgG IIF		
			French recommendations		Other country recommendations				
	Negative (<6 IU/ml)	Equivocal (6–9 IU/ml)	Negative (<1 IU/ml)	Equivocal (1–30 IU/ml)	Negative or equivocal (<3 IU/ml)	Positive (≥3 IU/ml)	Negative (<6)	Positive (≥6)	Not done
Positive	24	2	4	22	6	20	14	12	0
Negative	12	1	8	5	11	2	11	1	1

[a] IB results according to IgG routine methods and IgG-IIF.

(Roche Diagnostics, Meylan, France) (18) based on a recombinant surface antigen (p30 or SAG-1). According to the manufacturer, Elecsys Toxo IgM is positive if ≥1, negative if <0.8, and equivocal if ≥0.8 and <1; in France, Elecsys Toxo IgG is positive if ≥30 IU/ml, negative if <1 IU/ml, and equivocal if ≥1 and <30 IU/ml.

The additional methods performed for the study were as follows. (i) An IgM immunosorbent agglutination assay (Toxo-ISAgA IgM, bioMérieux, Marcy l'Etoile, France) (8) to confirm the specificity of IgM, thus preventing interpretation errors and confirming patient inclusion (12). According to the manufacturer, the test is positive if ≥9, negative if <6, and equivocal if ≥6 and <9. (ii) Platelia Toxo IgG and IgM (Bio-Rad, Marnes-la-Coquette, France), our routine methods, are indirect immunoenzymatic tests using a mixture of antigens (enzyme-linked immunosorbent assay [ELISA]) (11, 13). They are performed on an automated analyzer (Etimax; Diasorin). According to the manufacturer, Platelia Toxo IgG is positive if ≥9 IU/ml, equivocal if ≥6 and <9 IU/ml, and negative if <6 IU/ml); Platelia Toxo IgM is positive if ≥1, negative if <0.8, and equivocal if ≥0.8 and <1. (iii) An IIF, which is a classical reference method, was performed with an in-house antigen from the *Toxoplasma* RH strain and an anti-IgG conjugate (Bio-Rad). The test is positive if ≥6 IU/ml. (iv) The Toxo II IgG test (LDBio, Lyon, France) (IB) is a qualitative immunoblot test in which parasite antigens are separated by electrophoresis and transferred by electroblotting to nitrocellulose strips. The kits include ready-made, numbered, and lobed strips; a positive-control serum; and all liquid reagents. The resulting bands on the patient's strip are compared with the five characteristic bands (30, 31, 33, 40, and 45 kDa) of the positive-control strip. A positive result with IB was defined by the presence of at least three matching bands on the patient's strip, including the 30-kDa band (5).

We focused on the first serum following a toxonegative sample in which IgM could be detected but IgG was negative or equivocal by the two routine methods (Platelia and Elecsys Toxo IgG). These samples were further tested with IB and IIF. Then, if no method permitted seroconversion diagnosis on the first serum, we investigated a second serum taken 2 to 5 weeks later, and so forth, until seroconversion was observed by one of the methods (routine methods, IIF, or IB).

RESULTS

For all the sera, IgM specificity was confirmed by the immunosorbent agglutination assay. Using IB and the other assays, early seroconversion diagnosis was comparatively assessed.

Study of the first sera, IgM positive but IgG negative (or equivocal) by routine methods. Among the 39 initial sera from pregnant women with IgM alone, 36 (92.3%) were negative for IgG and 3 (7.7%) were equivocal for IgG using the Platelia test (Table 1). Of these same sera, 12 (30.8%) were negative and 27 (69.2%) equivocal using the Elecsys Toxo IgG test.

IIF permitted seroconversion diagnosis for 13 of the 39 sera (34.2%) where routine methods were negative or equivocal.

IB was able to detect the presence of *Toxoplasma*-specific IgG in 26 (66.7%) of the 39 initial sera, whereas routine methods were negative or equivocal (Table 1). Among these 26 cases IgG positive by IB, 12 (46.2%) were also identified as positive by IIF. Thus, IB identified seroconversion in an additional 14 patients (35.9%) for whom routine methods and IIF were negative. Among the 26 IB-positive cases, 10 had 3 bands (38.5%), 14 had 4 bands (53.8%), and 2 had 5 bands (7.7%). For the remaining 13 patients (IB IgG negative), no other methods (IIF and routine methods) permitted seroconversion diagnosis, except in one case where IIF alone was positive (Fig. 1).

Second-serum studies in the absence of seroconversion diagnosis on the first serum by IB. For the 13 undiagnosed cases (IB and routine methods were negative on the first serum; IIF results were not considered), a second sample was taken 2 to 5 weeks later. Seroconversion was detected in 10 patients (76.9%) by IB where routine methods remained negative; IIF was positive in 5 of these 10 cases (50%), negative in 2 (20%), and not done in 3 (30%). Seroconversion was detected in 1 patient (7.7%) by IB, Elecsys Toxo IgG, and IIF (the Platelia Toxo IgG test was negative) and in 1 patient (7.7%) by routine methods and IIF (IB was not done, as the routine methods were positive) and was not detected in 1 patient (7.7%) by IB, routine methods, or IIF. However, for the last patient, routine methods detected serum conversion in a third serum taken 3 weeks later (IB was not necessary).

Conclusions after study of initial and second-round sera. IB allowed an earlier toxoplasmosis seroconversion diagnosis in 92.3% of cases (36 of 39 cases), while the routine methods were still negative or borderline. Seroconversion was diagnosed simultaneously by IB and routine methods in only 2

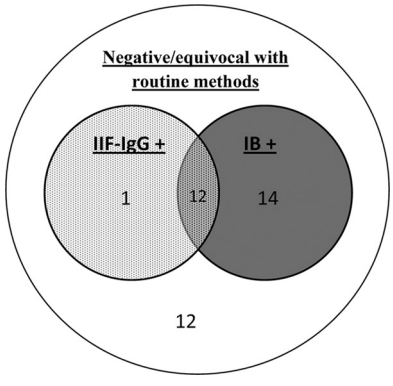

FIG. 1. Superiority of IB versus IIF-IgG for toxoplasmosis seroconversion diagnosis in first sera that are IgM-positive and IgG-negative or -equivocal by routine methods.

A

Date	IgG Platelia • Positive if ≥ 9 IU/mL • Equivocal 6-9 IU/mL	IgM Platelia • Positive if ≥ 1 • Equivocal 0,8-1	IgG Elecsys • Positive if ≥ 30 IU/mL • Equivocal 1-30 IU/mL	IgM Elecsys • Positive if ≥ 1 • Equivocal 0,8-1	IgG IIF • Positive if ≥ 6 • Negative if < 6	IB Positive if ≥ 3 bands including the 30 kDa band
07 Jan. 2010	0	0.2	< 0.13	0.16	0	Not done
16 Feb. 2010	1.0	3.9	0.81	5.59	2	30
20 Mar. 2010	4.6	3.1	13.7	4.07	2	30,31,33,45
09 Apr. 2010	11	3.1	Not done	Not done	6	Not done

B

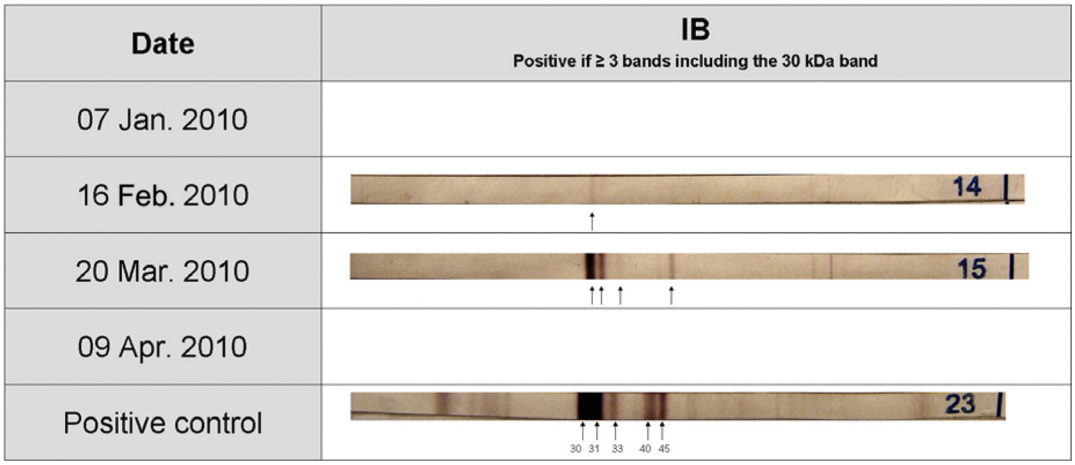

FIG. 2. Comparison of IB, Platelia Toxo IgG, Elecsys Toxo IgG, and IIF tests for sequential sera from one seroconversion in a 32-year-old pregnant woman. (A) Numerical results. (B) Evolution of immunoblot band appearance.

cases (5.1%). Comparing IB and IIF, the former was capable of correctly establishing 48.5% (16/33 cases) of seroconversion diagnoses while the latter was still negative. In one case (2.6%), neither IB, routine methods, or IIF permitted diagnosis. No false positives for IB were observed. IB demonstrated very good sensitivity for diagnosing seroconversion, particularly in the initial sera. The superiority of IB was less marked on second sera compared to IIF, but it still outperformed routine methods.

Order of band appearance in the Toxo II IgG test. Figure 2 shows the evolution of tests for a 32-year-old pregnant woman seronegative for toxoplasmosis before pregnancy (serology was dated 7 January 2010). On the sample taken 16 February 2010, we noted the appearance of IgM with Platelia and Elecsys IgM tests, but these routine methods, as well as IIF and IB (only the 30-kDa band was present), were negative for IgG detection. Five weeks later (20 March 2010), another serum sample was taken; IgM persisted, and IgG was still negative with Platelia Toxo IgG and IIF but equivocal with Elecsys Toxo IgG. Conversely, IB indicated seroconversion via the appearance of four bands (30, 31, 33, and 45 kDa). The seroconversion diagnosis was confirmed 3 weeks later (9 April 2010) by Platelia Toxo IgG and IIF. Thus, in this case, the appearance of the 30-kDa band in the Toxo II IgG test preceded all other indicators, suggesting the diagnosis of seroconversion 7 weeks ahead of routine methods and IIF.

On the first group of sera, among the 26 IB results allowing seroconversion diagnosis, the 30-kDa band, which is indispensable for IB positivity, was of course consistently present. The 40- and 45-kDa bands were the most frequently present (100% and 80.8% of cases, respectively), followed by the 31-kDa band (77%); the 33-kDa band appeared less frequently (11.5%) (Table 2).

For the 13 pregnant women whose seroconversion was not confirmed in the initial sera (routine methods and IB were negative), the appearance of bands was analyzed in the successive sera and compared to the first. Of note, in one case, IB

TABLE 2. Frequencies of IB band appearances according to the successive sera after seroconversion diagnosis

Sample in which IB bands were present	Band (kDa)	No./total no. (%) of patients[a]	
		Seroconversion diagnosis on 1st serum	Seroconversion diagnosis on 2nd serum
1st	30	26/26 (100)	7/12 (58.3)
	31	20/26 (77)	2/12 (16.7)
	33	3/26 (11.5)	0/12 (0)
	40	26/26 (100)	2/12 (16.7)
	45	21/26 (80)	1/12 (8.3)
2nd	30		11/11 (100)[b]
	31		8/11 (72.7)[b]
	33		2/11 (18.2)[b]
	40		9/11 (81.8)[b]
	45		10/11 (90.9)[b]

[a] For one patient, seroconversion diagnosis was made on the third serum by routine methods: bands at 30 and 31 kDa were present on the first serum and bands at 33 and 40 kDa on the second serum.

[b] Immunoblotting was not done for one patient.

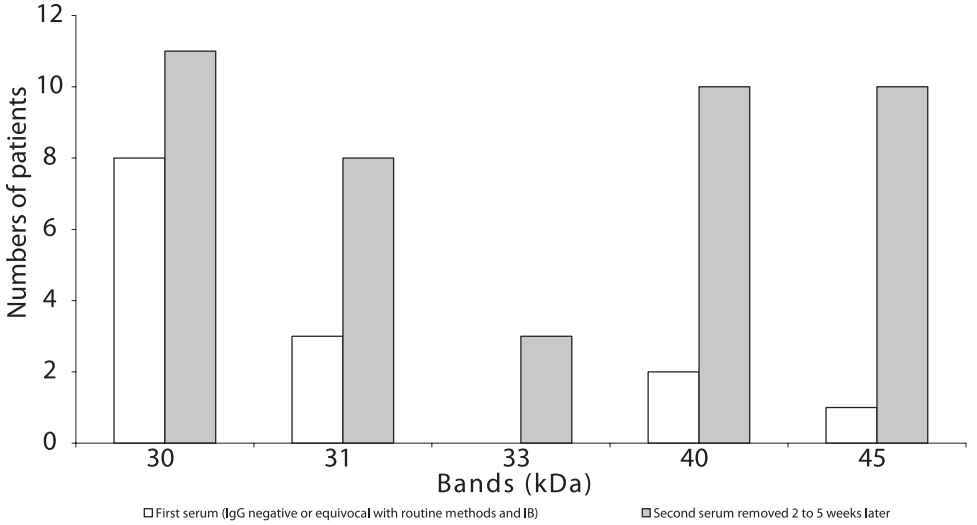

FIG. 3. Order of IB band appearance during seroconversion based on follow-up of 12 pregnant women whose first serology was IgM positive and IgG negative or equivocal by routine methods and IB.

was not performed on the second serum because routine methods were positive (Fig. 3). The IB 30-kDa band was already present on the first serum for 8 of these 13 women (61.5%), and another band was present for 5 of the 7 (71%); 4 of the 13 had any band, and 1 had a band other than the 30-kDa band. In the second sera, the 30-kDa band was consistently present, except in one case (IB negative). Thus, the appearance of the 30-kDa band was often a precursor of a coming seroconversion (Table 2). For the other bands, no particular order of appearance could be demonstrated.

DISCUSSION

Laboratories are faced with a number of result interpretation problems when using routine methods because of a lack of standardization and their large equivocal ranges (9). The positive threshold for Elecsys Toxo IgG is not clear and differs between countries: in France, the positive threshold is 30 IU/ml, whereas it is 3 IU/ml in other countries (15). In our study, the 3-IU/ml threshold for the Elecsys IgG test would have resulted in earlier seroconversion diagnoses for 22 of the 39 cases (56.4%) (Table 1). However, a recent study showed that equivocal results with Elecsys Toxo IgG (1 to 30 IU/ml) must be confirmed by IB due to specificity problems (11). Our study confirmed the lack of IgG detection by routine methods, although their association may be beneficial for early diagnosis of seroconversion.

The Toxo II IgG test was developed by LDBio to confirm serological test results for low titers of IgG. A previous study (5, 6) evaluated its use as a confirmatory test for at-risk patients (pregnant women, newborns, subjects with immune deficiencies, etc.), but only five pregnant women were included (99.2% sensitivity; 100% specificity). They compared the assays to the Sabin-Feldman dye test. The latter is a historical gold standard (developed in 1948), but it is also expensive, time-consuming, and difficult to standardize. Furthermore, the Sabin-Feldman dye test has largely fallen out of routine use (16).

In our prospective study, IB appeared to be an excellent alternative first-line seroconversion confirmation test in pregnant women: on two consecutively analyzed sera, it provided an earlier toxoplasmosis seroconversion diagnosis in 92.3% of cases (36/39). Thus, IB, which had lower sensitivity than immunoenzymatic methods in general immunology, can strongly detect IgG toxoplasmosis bands, whereas routine methods are negative. In the initial serum samples positive for IgM but negative or equivocal for IgG by routine methods, IB diagnosed seroconversion twice as often as IIF (26/39 [66.7%] versus 13/39 [33.3%]; $P < 0.001$; χ^2 test). IIF alone established a diagnosis in only one case (2.6%). In the second-serum samples, the superiority of IB was incontestable compared to routine methods but less clear compared to IIF. In practice, as days pass and the presumed seroconversion date fades into the past, antibody titers increase and thus become easier to detect. Our study shows that IB provides better performance than IIF, which is considered today to be the method offering the earliest detection of IgG. In fact, our results showed higher sensitivity of IIF than routine methods (Platelia and Elecsys IgG tests) (13/39 IIF positive versus 0/39 positive by routine methods). However, the complexity of IIF and the subjective nature of the interpretation of its results limit its routine use to specialized laboratories. A second exception to the superiority of IB was illustrated by the case of one patient (2.6%) for whom the diagnosis could be established neither in the first serum nor in the second by IB (IIF and routine methods were also negative). For this patient, 26 days (and a third sample) were necessary to establish the seroconversion diagnosis, although it should be kept in mind that initiation of treatment may have delayed the appearance of IgG (3, 6).

Analysis of the order of band appearance demonstrated that the 30-kDa band tends to appear first. The 30-kDa band was already present in 33 (91.7%) of the 39 initial sera that were IgM positive and IgG negative or equivocal by routine methods; seroconversion was confirmed for all of them in the second sera. Furthermore, the presence of the 30-kDa band per-

mitted seroconversion diagnosis in 20 of the 33 cases (60.6%), while IIF was still negative. Thus, the appearance of the 30-kDa band on the first serum may allow physicians to diagnose seroconversion without waiting for another serum. The Toxo II IgG test is easily carried out and interpreted; it can be implemented in specialized and nonspecialized laboratories. No false positives were reported with IB. The ability of this sensitive test to confirm seroconversion weeks ahead of other tests could help in reducing costly monitoring of pregnant women and allow earlier infection management and treatment. However, it should be noted that our study provides no information on the postseroconversion follow-up of the pregnancy or the newborn.

The French congenital-toxoplasmosis screening program is built principally upon serological testing and monitoring of pregnant women with negative serology at the start of pregnancy. The aim of the program is to establish an early diagnosis and provision of treatment for cases of obstetrical seroconversion. Our study confirmed the reference range for the Toxo II IgG test, which also provided better overall results than other commercial tests. Our study strongly suggests that the Toxo II IgG test may enhance toxoplasmosis care in pregnancy by reducing the time to therapeutic intervention and thus disease-related sequelae in children. It may also reduce health care expenses by eliminating the need for further toxoplasmosis serological monitoring.

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