Use of IgG in Oral Fluid To Monitor Infants with Suspected Congenital Toxoplasmosis

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Infants born to mothers who seroconverted for toxoplasmosis during pregnancy are at risk of sequelae. In the case of a negative work-up at birth, congenital infection can be ruled out only by monitoring the disappearance of maternal immunoglobulin G (IgG) transmitted through the placenta, which can be achieved by regular blood sampling during the first year. To alleviate the discomfort of this follow-up, we developed an indirect enzyme-linked immunosorbent assay to detect specific IgG diffusing passively from the blood through the gingival epithelium by collecting oral fluid on microsponges. To assess the feasibility of the test, 212 patients were first enrolled. Levels of specific IgG in oral fluid were significantly higher in seropositive (n = 195) than in seronegative (n = 17) patients (mean optical densities, 1.145 ± 0.99 versus 0.092 ± 0.127; P < 0.0001). In a population of 93 patients <15 months of age born to mothers who displayed toxoplastic infection during pregnancy, 70 were free of congenital infection and were followed up until their serology turned negative, and 23 were congenitally infected. The same patterns of IgG were observed in the oral fluid and sera in each group. Using a cutoff of 0.04 (optical density value), the sensitivity and specificity of the test were 67.9% and 80.3%, respectively, and the probability of not having a congenital infection when the test on oral fluid was negative was 99%. Although the performance of the test needs to be improved, oral fluid sampling appears to be a promising tool for monitoring infants with suspected congenital toxoplasmosis.

Toxoplasma gondii is a worldwide obligate intracellular protozoan parasite that causes toxoplasmosis, which usually occurs without symptoms. However, serious manifestations may occur in immunocompromised patients or in fetuses. The clinical presentation of congenital infection ranges from fetal loss to severe neurologic or ocular lesions to subclinical infection (1), from which infants can develop retinal diseases during childhood or adolescence (2). In France, due to prenatal mass screening for toxoplasmosis in pregnant women, each newborn from a mother who presents with toxoplasmosis during pregnancy undergoes a complete work-up at birth, including a funduscopic examination, cranial ultrasonography, and serologic tests for specific immunoglobulin M (IgM), IgA, and IgG. Because antenatal and perinatal work-ups do not provide a sensitivity of 100% when the results are negative, congenital infection cannot be ruled out completely. Maternal IgG crosses the placenta, and its presence in the serum of newborns cannot be considered to be a marker of congenital infection. The universally accepted reference standard for ruling out a congenital T. gondii infection is a negative test for specific IgG within the first year of life, which demonstrates that the infant has not secreted IgG and has totally eliminated the maternal antibodies (3). This can be achieved only through regular blood sampling during that first year, which is not well accepted by children or parents and requires trained personnel. In some settings, such as France, all maternal infections are detected through the mass screening of nonimmunized pregnant women. In one study, 75% of children born to women who seroconverted during pregnancy were free of infection (4), but they needed to be tested regularly. To improve compliance with the follow-up, it is important to reduce the burden and discomfort that this testing can cause, and oral fluid appears to be an appropriate noninvasive means for following the decline of IgG titers. Oral fluid is a mixture that includes secretions from the salivary glands, gingival crevice fluid, and bronchial and nasal secretions (5). It contains secretory IgA that is synthesized by the salivary glands and IgG and IgM that are derived from serum exudates from capillaries along the gum. The three major antibodies, as well as most components of the blood, can be detected in oral fluid at lower concentrations (6). Several studies have successfully investigated the use of oral fluid or saliva versus serum for the diagnosis of infectious diseases, including infections with HIV (7), hepatitis A virus (8), dengue virus (9), Helicobacter pylori (10), and malaria (11). In addition, antigens, such as hepatitis B surface antigen (12) and Plasmodium falciparum HRP2 malaria antigen (13), and hormones, such as steroids (14), have been assayed in oral fluid. In the field of toxoplasmosis,
some authors have already reported the possibility of detecting anti-\textit{T. gondii} IgG (15, 16), IgM, and IgA (17, 18). The goal of this study was to investigate the feasibility and accuracy of the detection of toxoplasma-specific IgG in oral fluid as an alternative to blood sampling for the follow-up of infants with suspected congenital toxoplasmosis.

**MATERIALS AND METHODS**

**Patients.** Four hospitals participated in the study, the Hôpital de la Croix Rousse (Lyon, France), the Policlinico San Mateo (Pavia, Italy), the Institut de Puériculture et Périnatalogie (Paris, France), and the Assistance Publique des Hôpitaux de Marseille (Marseille, France). The study was carried out on patients at risk of congenital infection who were referred to outpatient departments for serology for \textit{Toxoplasma} and patients who were free of toxoplasmic infection but were hospitalized for other reasons and served as negative controls (Fig. 1). A total of 322 patients was included in the study. The first group included 212 patients comprising 108 pregnant and nonpregnant women (age range, 19 to 53 years) and 104 children and adolescents (age range, 16 months to 18 years) who were enrolled in a pilot study to check the validity of the sampling procedure (in terms of feasibility and with respect to the volume of saliva to be collected) and to develop an appropriate enzyme-linked immunosorbent assay (ELISA). A second group, which represented our target population, comprised 110 infants <15 months of age who were enrolled prospectively; 93 of them were born to women who seroconverted during pregnancy, and the remaining subset of 17 hospitalized infants who were not at risk for congenital toxoplasmosis (born to mothers with negative serology at delivery) was enrolled as a control group. For infants at risk, congenital toxoplasmosis work-ups at birth (imaging and specific serum IgM and IgA assays) was negative in 70 patients and positive in 23 cases. All of these patients were followed for at least 1 year, depending on their serologic status.

**Sample collection.** Oral fluid was collected using two microsponges (Beaver-Visitec, Waltham, MA, USA) placed between the lower gum and the cheek for at least 2 min. The microsponges were placed in swab storage tubes (Salimetrics, State College, PA, USA) (Fig. 2) and kept at 4°C. Within 2 h after sampling, the oral fluid was centrifuged at 3,000 × g for 15 min at 4°C and stored or shipped at −20°C until use. Peripheral blood was drawn into Vacutainer SST tubes (BD Diagnostics, Franklin Lakes, NJ, USA) containing spray-coated silica and a polymer gel.

**Enzyme-linked immunosorbent assay.** Salivary anti-\textit{T. gondii} IgG was detected by an in-house indirect enzyme-linked immunosorbent assay (ELISA). Briefly, inactivated \textit{T. gondii} antigen-precoated strips (Enzygnost Toxoplasmosis/IgG; Siemens Healthcare Diagnostics, Marburg, Germany) were blocked with 200 μl of 0.5% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) for 2 h at room temperature. The strips were washed four times with PBS-T. The oral fluid samples were thawed and centrifuged a second time at 3,000 × g for 15 min at 4°C to remove potential debris. Either 50 μl of oral fluid or 50 μl of PBS (blank) was incubated at 37°C for 1 h. After a washing cycle with PBS-T, 200 μl of monoclonal anti-human IgG biotin conjugate (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:60,000 in PBS-T was added. The strips were incubated for 2 h at room temperature and then washed four times with PBS-T. Wells were incubated with 200 μl of 1/8,000 ultrasensitive streptavidin-peroxidase polymer (Sigma-Aldrich). Following a washing cycle with PBS-T, reactions were developed with 100 μl per well of 3,3’5,5’-tetramethylbenzidine (TMB) (Sigma-Aldrich). Reactions were stopped by 100 μl of 1 N hydrochloric acid after incuba-
tion for 7 min in the dark at room temperature. The optical density (OD) was measured at 450 nm (reference, 650 nm). Each experiment included a blank and a positive and a negative sample. The OD of the blank was subtracted from the ODs of the samples. All tests were performed at the Hôpital de la Croix Rousse (Lyon, France).

Serum anti-

*T. gondii* IgG was tested using the AxSYM Toxo IgG microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL, USA). Patients aged 15 months of age with 3 IU/ml of serum IgG were considered to be negative. In infants and newborns aged 15 months of age, the cutoff was adjusted to 1.3 IU/ml.

Statistical analysis. The statistical analysis was performed using VassarStats (http://vassarstats.net/). The sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio of the salivary anti-

*T. gondii* IgG ELISA were calculated using the AxSYM Toxo IgG microparticle enzyme immunoassay as the reference standard. The t test was used to compare the salivary IgG OD of the *T. gondii-*seropositive and *T. gondii-*seronegative patients. Kernel regression smoothing for time series was used to describe and compare the evolution of the oral fluid and serum anti-

*T. gondii* IgG. The receiver operating characteristic (ROC) curve was used to demonstrate the evolution of sensitivity and specificity in relation to the thresholds chosen to classify the oral fluid test as positive or negative. These two analyses were performed in the R version 3.1.1 program environment (R Foundation for Statistical Analysis).

Ethical aspects. This study was approved by the local ethical committee (Comité de protection des personnes Sud Est II), and informed consent was obtained from each patient or his or her legal guardian(s).

RESULTS

Pilot study. Figure 3 shows that the levels of anti-

*T. gondii* IgG in oral fluid were significantly higher in seropositive (n = 195) than in seronegative (n = 17) patients (mean ODs, 1.145 ± 0.999 versus 0.092 ± 0.127, respectively; *P* < 0.0001). In addition, a positive relationship was observed between the levels of anti-

*T. gondii* IgG in oral fluid and in sera (*r* = 0.4516; *P* < 0.0001).

Study of newborns and infants younger than 15 months. The levels of serum and oral fluid IgG after repeated tests were compared among a group of 70 (220 samples) noninfected newborns during their first year of life until their serology scored negative. These infants presented a negative antenatal and/or a negative perinatal work-up and did not receive treatment for toxoplasmosis, and their serology became negative within the first year. No failure to sample oral fluid was observed. Microsponges appeared to be safe and were well accepted by the infants. The sampling time was shortened to 30 s when the patient salivated sufficiently, and an average volume of 230 l per sample was collected. When necessary, microsponges were withdrawn and placed in the mouth again. A positive correlation was observed between the two sets of samples (*r* = 0.598; *P* < 0.0001).

The patterns of decreased IgG levels over time in the two assays were compared using nonparametric kernel regression smoothing for time series (see Fig. 4). The line of the moving average of each value (this line smooths irregularities and allows the recognition of trends in the time series) displayed comparable profiles in the two subsets of data, demonstrating that maternally specific IgG
levels were eliminated according to the same profiles from sera and oral fluid. Figure 5 shows a comparison of the results of the simultaneous detection of paired samples of oral fluid and sera in three noninfected infants who were followed for 11, 13, and 14 months, respectively. An ROC curve (Fig. 6) was produced for the specific IgG levels in oral fluid corresponding to the first negative serologic test when follow-up was discontinued and congenital infection was ruled out. Table 1 shows the sensitivities, specificities, positive likelihood ratios, negative likelihood ratios, and the probabilities of the presence or absence of congenital toxoplasmosis according to four different cutoff points in the oral fluid IgG assay chosen according to the ROC curve.

From our cohort of infants born to women who were infected during pregnancy, the probability of a false-negative work-up at birth was estimated to be 2%. Given this pretest probability in our clinical setting, a cutoff of 0.04 (optical density value) scored a probability of 99% for the absence of congenital toxoplasmosis in the case of a negative test and a probability of 6% for the presence of congenital toxoplasmosis in the case of a positive test. The 23 congenitally infected patients were treated and followed up according to our protocol (19). Their levels of specific IgG followed the same profiles in oral fluid and in sera, with the exception of those for one patient who displayed a negative oral fluid test despite a positive serologic score (data not shown). In the group of 17 infants who were not at risk for congenital toxoplasmosis, nine scored above the cutoff, and two of them displayed ODs of 1.14 and 1.71.

**DISCUSSION**

We demonstrated that monitoring infants with suspected congenital toxoplasmosis can be achieved through the detection of IgG in oral fluid.
This test requires only a small volume of oral fluid (50 μl), which was easily obtainable from all the patients. Sampling was performed without problems, and no difficulties were experienced in handling the microsponges. The time of collection with respect to meals, smoking, and alcohol consumption did not alter the results (data not shown). As no preservatives were added, the oral fluid had to be frozen within 2 h after sampling in order to protect the Ig from proteases. Whether an additive would allow a longer delay between the sampling and processing of oral fluid should be investigated. Data obtained from the pilot study showed that an adapted commercialized microplate ELISA kit can detect a significant difference in the IgG in oral fluid between seropositive and seronegative patients (Fig. 3). The dispersion of OD values reflected the wide range of serologic IgG titers in this unselected population. In the pilot study and the group of infants <15 months of age, we observed significant correlations between IgG levels in sera and oral fluid ($r = 0.456$, $P < 0.0001$, and $r = 0.598$, $P < 0.0001$, respectively). A similar correlation was also reported for other infectious diseases, such as malaria (11), tetanus antitoxin (19), rubella (20), and dengue (for IgA) (9). In our target population of 70 uninfected infants <15 months of age, a parallel decline in IgG was observed in the two biological fluids, and the line of the moving average illustrated the similarity in the patterns of IgG evolution in oral fluid and sera (Fig. 4). In addition, Fig. 5 presents strikingly similar patterns in the disappearance of IgG in three uninfected infants born to mothers who seroconverted during pregnancy. In this clinical setting, the goal is to confirm the disappearance of maternal IgG to rule out congenital infection. Therefore, false-negative results must imperatively be avoided. In a subgroup of 23 infants treated for congenital toxoplasmosis, concordant IgG patterns between sera and oral fluid were observed in all but one sample, which displayed a false-negative result for oral fluid. During the follow-up of treated infants, serologic IgG titers often displayed transitory negativity (21), followed by a relapse after drug withdrawal (22). A similar pattern was also observed in oral fluids (data not shown). Table 1 shows the performance of the test according to four different cutoffs. The performance of the test did not change significantly according to the different values. Interestingly, a negative score had a high probability for the absence of congenital toxoplasmosis. Conversely, a positive test was associated with a very low probability for the presence of the disease. The low sensitivity observed (Table 1) may be due to the concentration of IgG in oral fluid being 10-fold lower than that in sera (23). Insufficient quantities of IgG due to inadequate sample collection were unlikely because the fluids were collected under the same conditions by doctors who followed the same protocol. The degradation of IgG by either bacteria or proteolytic enzymes was prevented by rapid freezing after sampling. Conversely, five patients showed positive results for IgG in oral fluid despite having negative serologic tests; two of them had been breastfed shortly before sampling. Of the remaining three, one presented with otitis and one presented with bronchitis when they were sampled. Among the 17 control infants who were free of congenital infection with negative serology at birth, nine scored above the cutoff and two had a high OD. These patients had been admitted for meconium inhalation and prematurity or for bacterial infection. Whether current infection can induce false-positive results remains to be investigated. False-positive results have already been observed in a test for HIV in oral fluids in a cluster of patients but have also remained unexplained (24). Taken together, these data demonstrate a good parallel in the evolution of IgG titers in oral fluid and sera. The detection of specific toxoplasmatic Ig (mainly IgG but also IgM and IgA) using different techniques has already been reported in adults and yielded sensitivities and specificities ranging from 25% to 98.5% and 94.12% to 100%, respectively (15, 16, 17, 18), but no other study has investigated the feasibility of such tests in newborns and infants. Although the performance of the test should be improved by investigating cross-reactivity and increasing its sensitivity, oral fluid sampling appears to meet all of the requirements for monitoring newborns and infants with suspected congenital toxoplasmosis. This noninvasive test not only may reduce the cost of follow-up (23) and increase its compliance but also can be performed easily (even by untrained staff) and prevent accidental exposure to blood. Parents were eager to see this routine sampling process replace blood testing.

**TABLE 1** Test parameters according to four different cutoff points of specific IgG in the oral fluid test

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result at cutoff (OD) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>68</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>80.3</td>
</tr>
<tr>
<td>Positive likelihood ratio</td>
<td>3.44</td>
</tr>
<tr>
<td>Negative likelihood ratio</td>
<td>0.399</td>
</tr>
<tr>
<td>Probability of absence of CT with a negative test (%)</td>
<td>99</td>
</tr>
<tr>
<td>Probability of congenital CT with a positive test (%)</td>
<td>6</td>
</tr>
</tbody>
</table>

*CT, congenital toxoplasmosis.*
ACKNOWLEDGMENT
We declare no conflicts of interest.

REFERENCES