Multicentric Evaluation of the Bio-Evolution *Toxoplasma gondii* Assay for the Detection of *Toxoplasma* DNA in Immunocompromised Patients

Nawel Ait Ammar, a,b Hélène Yera, c Jeanne Bigot, d Françoise Botterel, a,b Christophe Hennequin, d Juliette Guitard d

a EA Dynamyc 7380 UPEC, ENVA, Faculté de Médecine de Créteil, Créteil, France
b Unité de Parasitologie-Mycologie, Département de Virologie, Bactériologie-Hygiène, Mycologie-Parasitologie, DIU VIC, CHU Henri Mondor, AP-HP, Créteil, France
c Laboratory of Parasitology-Mycology, Université Paris Descartes, AP-HP, Hôpitaux Universitaires Paris Centre, Hôpital Cochin, Paris, France
d Centre de Recherche Saint-Antoine, CRSA, AP-HP, Sorbonne Université, Inserm, Hôpital Saint-Antoine, Paris, France

**ABSTRACT**

PCR-based methods are a key tool for the diagnosis of toxoplasmosis in immunocompromised patients. Laboratory-developed protocols lack standardization. This study aimed to assess the performances of a commercial kit for the detection of *Toxoplasma* DNA in different specimens drawn from immunocompromised patients. This multicentric retrospective study included 227 DNA specimens (157 blood specimens, 22 bronchoalveolar fluid [BALF] specimens, 39 cerebrospinal fluid [CSF] specimens, and 9 miscellaneous specimens) collected between 2010 and 2015 from 126 immunocompromised patients. The specimens were selected based on previous laboratory-developed quantitative PCR (qPCR) analyses targeting either the rep529 element or the *B1* gene, and the results were classified as positive, negative, and “negative of interest,” where the latter was defined as representing either the last specimen with a negative result before a positive one or the first with a negative result following a positive result(s). All specimens were secondary tested using the Bio-Evolution *Toxoplasma* DNA assay targeting the *T. gondii* rep529 element. We found a 95.6% concordance rate for qualitative results obtained with laboratory-developed qPCR techniques and the commercial kit. The rate reached 99.3% in comparisons of rep529-based laboratory-developed PCR methods and the commercial kit. The quantifications obtained with the commercial kit and the rep529 laboratory-developed PCRs were in very good agreement. Sensitivity and specificity of the commercial kit were calculated at 98.8% and 100%, respectively. The Bio-Evolution *Toxoplasma* DNA assay appears to be a valuable method for the detection of *Toxoplasma* DNA in blood, BALF, and CSF specimens from immunocompromised patients.

**KEYWORDS** toxoplasmosis, quantitative PCR, immunocompromised patients

Toxoplasmosis is a severe complication under various conditions of immune deficiency, such as AIDS, solid-organ transplantations (SOT), and allo-bone marrow transplantation (allo-BMT) (1). In hematologic patients, the management of *Toxoplasma* infection is particularly challenging because first-line therapy, which relies on the combination of sulfamethoxazole and trimetoprim, is myelotoxic (1) and so can be deleterious for the engraftment (2, 3). Thus, sensitive and specific diagnostic tools are required to establish a rapid and reliable diagnosis to anticipate application of a dedicated therapy or to rule out a diagnosis, thus avoiding unnecessary toxic therapy. Clinical symptoms greatly vary, including asymptomatic to isolated fever, pulmonary signs, and/or neurological symptoms (4). Imaging data (computed tomography [CT] scanning, magnetic resonance imaging [MRI]) support the diagnosis when multiple lesions with mild perifocal edema are detected in the brain. However, detection of...
those lesions is not sufficiently specific to enable a definitive diagnosis (5). Because toxoplasmosis in immunocompromised patients mostly results from reactivation of latent infection, serological tests are useless in that they can confirm previous immunization only. Demonstration of the presence of *Toxoplasma* trophozoites in biological specimens such as bronchoalveolar fluid (BALF) or cerebrospinal fluid (CSF) specimens is unusual. Thus, molecular techniques designed to detect *Toxoplasma* DNA have emerged as the most suitable methods for the diagnosis of toxoplasmosis in immunocompromised patients (6). Indeed, molecular methods allowed the detection of 89% of the toxoplasmosis cases in a large population of immunocompromised patients and were thus the most effective method for the diagnosis (4). Nevertheless, it should be kept in mind that the sensitivity of those tests depends on the type of specimens analyzed whereas their specificity does not enable distinguishing between disease (presence of suggestive symptoms) and infection (asymptomatic patients) (7, 8). In many medical centers, PCR has now been adopted as a screening test for toxoplasmosis in patients at higher risk, particularly allo-BMT patients, who were found to represent 66% of the transplant patients with toxoplasmosis (4).

Until recently, the *B*1 gene (9) and the rep529 noncoding DNA element (10) (GenBank accession no. AF146527) were the most commonly targeted loci (11). Currently, most of the laboratories use in-house-developed tests, leading to a lack of standardization as each laboratory-developed test has been set up independently (11). Currently, the number of commercial kits to detect *Toxoplasma gondii* DNA is still limited and their routine performances have been rarely evaluated. The Bio-Evolution *Toxoplasma gondii* assay has been favorably assessed for the diagnosis of congenital toxoplasmosis (12) but had not yet been applied to diagnose toxoplasmosis in immunocompromised patients.

This study aimed to assess the value of this commercial kit for the detection of *Toxoplasma* DNA in clinical (CSF, BALF, and blood) specimens collected from immunocompromised patients.

**MATERIALS AND METHODS**

**Study design.** This multicenter study involved the Parasitology and Mycology departments of 3 French University hospitals in Paris, France. All 3 centers were asked to retrieve DNA samples that were tested previously (2010 to 2015) for *Toxoplasma* DNA using their own laboratory-developed *Toxoplasma* quantitative PCR (qPCR) method. These DNAs had been extracted from BALF, CSF, and blood specimens drawn from immunocompromised patients at risk of disseminated toxoplasmosis and were stored at –20°C. Patients were considered at risk of disseminated toxoplasmosis if they were seropositive for *T. gondii* and if they were immunocompromised due to HIV infection, hematological malignancy, allo-BMT, and SOT. According to initial qPCR results and clinicoradiological data, the patients had been diagnosed either free of *Toxoplasma* infection (n = 81) or, using previous definitions, with either a *Toxoplasma* disease (n = 34) or a *Toxoplasma* infection (n = 11) (8). Furthermore, we classified specimens as positive, negative, or “negative of interest,” the latter corresponding either to the last negative specimen result before a positive specimen result or the first negative specimen result after a positive specimen result.

**qPCR methods.** For the blood specimens, DNA had been extracted from whole-blood specimens in two centers and buffy coat specimens in the third (13). DNA extraction from all specimens has been performed using a QIAamp DNA minikit (Qiagen).

Each center used its own laboratory qPCR protocol. Two of them performed amplification of the rep529 element, testing the samples either in triplicate (14) or in duplicate (15). The third used a qPCR method targeting the *B*1 region in simplicate (16). In all the cases, a test was considered positive if the PCR crossing point (Cp) value was <40. Inhibition and extraction controls were used for each specimen in all the laboratories. Detailed technical notes are provided in Table 1.

Each center performed the commercial qPCR using its specimens. Commercial kits were provided at no cost by BioEvolution. The Bio-Evolution kit consists of a ready-to-use real-time PCR based on a hydrolysis technology using a *T. gondii* probe targeting the rep529 genomic element. An internal control is added to the PCR mix to detect PCR inhibitor. In our study, DNA specimens were first tested in simplicate. In the case of a qualitative discordance between laboratory-developed PCR and the Bio-Evolution assay, the given specimen was tested again in simplicate with the Bio-Evolution kit.

**Statistical analysis.** Data were analyzed using Prism V7.0a software (GraphPad Software). Pearson coefficient values were calculated to test the correlations between pairs of quantitative measures. Semi-quantitative results determined for different groups of patients were compared in a Bland-Altman plot. Multiple comparisons were calculated using the Tukey’s test as appropriate. A *P* value of <0.05 was considered significant.

**Ethics considerations.** All procedures contributing to this work complied with the ethical standards of the French Ethics Committee on human experimentation and with the Helsinki Declaration of 1975.
as revised in 2008. Since the study was performed retrospectively on specimens collected through routine clinical tests and since patient-identifiable information was anonymized, no written or verbal informed consent to participate in this study from the patient was necessary. We had no contact or interaction with the patients.

RESULTS AND DISCUSSION

To the best of our knowledge, there is no published study which focused on the assessment of commercial kit for the detection of Toxoplasma DNA in immunocompromised patients. Here, we compare the results previously obtained in 3 different centers, using different laboratory-developed protocols, to those obtained with the Bio-Evolution commercial kit.

Description of patients and specimens. BALF, CSF, and blood specimens were collected from immunocompromised patients at risk of disseminated toxoplasmosis. Patients were already diagnosed using the laboratory-developed PCRs either as “free of toxoplasmosis” (all-specimens negative for Toxoplasma PCR) (n = 81) or as suffering from Toxoplasma disease (n = 34) or Toxoplasma infection (n = 11). Patients with Toxoplasma disease presented mainly with cerebral symptoms (n = 24) or pulmonary symptoms (n = 4) or both (n = 6). Clinical data were lacking for 25 Toxoplasma-PCR positive patients (26 specimens). A total of 227 specimens consisting of 157 blood specimens, 22 BALF specimens, 39 CSF specimens, 4 biopsy specimens (3 cerebral and 1 pulmonary specimens), 4 bone marrow aspirate specimens, and 1 aqueous humor specimen were gathered and categorized as positive (n = 91), negative of interest (n = 30), and negative (n = 106). A detailed description of patients and specimens are available in Table S1 in the supplemental material.

Qualitative correlation between laboratory-developed PCRs and Bio-Evolution kit. All specimens found negative with the laboratory-developed protocols and collected from patients who never developed Toxoplasma infection or Toxoplasma disease returned a negative result with the Bio-Evolution kit, resulting in 100% specificity.

All specimens but 5 that tested positive with one of the laboratory-developed PCR methods were found positive with the Bio-Evolution assay (Table 2). In the cases of discrepancy (false-negative results), only 3 specimens were available for a second run of testing with the Bio-Evolution test. Two of them returned positive results whereas another remained negative. For the latter specimen, a very low parasitic load had been detected with the laboratory-developed PCR targeting rep529 as only 1 of 3 triplicates returned positive with a Cp value at 39.7.

Finally, five negative of interest specimens (corresponding for 1 to the last negative specimen result before a positive specimen result and for 4 to the first negative specimen result after a positive specimen result) were found positive with the Bio-Evolution kit with Cp values of 31.12, 35.4, 35.99, 36.84, and 37.29. Due to the limited
volume of sample, we were able to retest and confirm the results with the Bio-Evolution kit for four of these specimens. In all 5 of these cases, the specimens had been previously tested with the \textit{B1} gene-targeting laboratory-developed PCR.

Overall, the concordance between the laboratory-developed PCRs and the Bio-Evolution kit was high, with the significance value calculated at 95.6\% ($P < 0.0001$). This value increased to 97.3\% ($P < 0.0001$) when the false-negative results were corrected after the second run of Bio-Evolution testing (Table 2). When we focused on the comparison of the results obtained only with the rep529-targeting laboratory-developed PCRs and the Bio-Evolution assay, including the results obtained after a second run addressing the discrepancies ($n = 138$), we retrieved a 99.3\% rate of concordance. These results are in agreement with those previously published by Filisetti et al., who found a 99\% concordance between the in-house-developed methods and the Bio-Evolution kit for the detection of \textit{Toxoplasma} DNA in amniotic fluid (12). In our study, the sensitivity of the method targeting the \textit{B1} gene was 82.7\%, considering the Bio-Evolution kit to be the reference. This shows that the \textit{B1} gene is far from optimal for the detection of \textit{T. gondii} genome. This may be due to its having a lower number of repeats (3 to 20 times fewer repeats according to the \textit{T. gondii} genotypes) than the rep529 region; the latter is now considered the reference for testing (17–20).

**Comparisons of semiquantitative results.** Comparisons of the semiquantitative results obtained with laboratory-developed PCR methods targeting the rep529 region and the Bio-Evolution kit for positive specimens ($n = 63$) are presented in a Bland-Altman plot (Fig. 1). This shows that only 5 specimens were excluded from the 95%-limit-of-agreement range (3 higher values and 2 lower values using the Bio-Evolution technique), indicating excellent agreement between the techniques. At this time, such differences do not have any impact on the therapeutic management of patients even if one can imagine the use of semiquantitative values for follow-up of those patients. A similar conclusion had been drawn for the diagnosis of congenital toxoplasmosis (12). A limit of our study was that we could not perform amplification with the laboratory-developed methods and the Bio-Evolution assay at the same time, so we cannot exclude the possibility of DNA degradation.

**Clinical performance of Bio-Evolution assay.** Data representing the clinical performance of the Bio-Evolution test are presented in Table 3. Clinical data were available for 30 HIV-positive patients, and all but one (97\%) presented with \textit{T. gondii} disease. In those patients, the clinical sensitivity of the Bio-Evolution assay was 100\%. At the time of diagnosis, the Cp median values determined with the Bio-Evolution kit were 32.68 (range, 26.07 to 38) and 26.55 (range, 23.94 to 38.3) for the CSF specimens ($n = 18$) and blood specimens ($n = 8$), respectively. Of note, all three patients with a positive qPCR result from the CSF samples had a concomitantly positive qPCR result from the blood samples. Clinical data were available for 15 HIV-negative patients. Five (33\%) of these patients presented with \textit{T. gondii} disease, while the other 10 had \textit{T. gondii} infection. One of them (presenting with pulmonary symptoms) would have been misdiagnosed using

### Table 2: Qualitative correlation between results obtained with 3 laboratory-developed qPCR methods and the Bio-Evolution kit for the detection of \textit{Toxoplasma gondii} DNA

<table>
<thead>
<tr>
<th>Result by laboratory-developed techniques</th>
<th>Bio-Evolution result</th>
<th>Cumulative no. of positive samples after first/second run</th>
<th>Cumulative no. of negative samples after first/second run</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive specimens ($n = 91/89$)</td>
<td>Bio-Evolution result</td>
<td>86/88</td>
<td>5/1</td>
</tr>
<tr>
<td>Negative of interest* ($n = 30$)</td>
<td></td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Negative specimens ($n = 106$)</td>
<td></td>
<td>0</td>
<td>106</td>
</tr>
</tbody>
</table>

*Two specimens could not be tested in a second run using the Bio-Evolution kit due to insufficient volume.

*Three of five specimens were available for retesting. One remained negative, while the other two turned positive.

See definition in Materials and Methods.
the Bio-Evolution assay, leading to a clinical sensitivity of 93.3%. In this case, a BALF specimen had been found positive in 1 of 3 triplicates with a laboratory-developed rep529 PCR, suggesting a very low parasitic load. In this group of patients, toxoplasmosis was predominantly diagnosed based on a positive result from the blood specimen (n = 12).

Gathering these results and considering the results of the laboratory-developed methods as the reference, we calculated the sensitivity and specificity of the Bio-Evolution kit at 98.8% and 100%, respectively. The likelihood ratios were 0.01 for a negative result and tended toward infinity for a positive result, confirming the reliability of the test either to support or to exclude the diagnosis of toxoplasmosis in immunocompromised patients. Whatever the underlying condition, we did not notice any significant differences in the Cp values that could be used as predictive of the clinical

**TABLE 3** Performance of Bio-Evolution assay at diagnosis in immunosuppressed patients who were either HIV positive or HIV negative

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value(s)</th>
<th>HIV-positive patients (n = 30)</th>
<th>HIV-negative patients (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients with clinical data available</td>
<td>30</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>No. of patients with T. gondii disease</td>
<td>29</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>No. of patients with T. gondii infection</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>% Bio-Evolution sensitivity</td>
<td>100</td>
<td>93.3</td>
<td></td>
</tr>
<tr>
<td>% Bio-Evolution specificity</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Median (range) Cp at diagnosis according to the specimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>26.55 (23.94–38.3) (n = 8)</td>
<td>33.12 (28.09–38.13) (n = 13)</td>
<td></td>
</tr>
<tr>
<td>BAL</td>
<td>27.45 (25.1–29.8) (n = 2)</td>
<td>20.6 (&gt;40) (n = 2)</td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>32.68 (26.07–38) (n = 18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebral biopsy</td>
<td>32.62 (31.09–34.15) (n = 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow aspirate</td>
<td>22.43 (n = 1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aGathering results from HIV-positive and HIV-negative patients led to global sensitivity of 98.8% and global specificity of 100%. The gold standard for diagnosis of toxoplasmosis consisted of clinical data, imaging, and result of laboratory-developed assays.*
form (disease versus infection) or the outcome (worsening versus resolution) (see Fig. S1 in the supplemental material). However, in patients with T. gondii disease, there was a trend toward a higher parasitic load in blood samples from HIV-positive patients (mean Cp = 27.8; range, 24.48 to 38.3) than in blood samples from HIV-negative patients (mean Cp = 37.7; range, 32.75 to 38.13) (Tukey’s multiple comparison; P = 0.0517) (Fig. S2).

In conclusion, with sensitivity of 98.8% and specificity of 100%, the Bio-Evolution kit appears to be a valuable alternative for use in detection of Toxoplasma gondii DNA in blood, BALF, and CSF specimens drawn from immunocompromised patients if 2 assays of Bio-Evolution are performed. Our qualitative results correlate perfectly with results obtained with laboratory-developed PCR targeting the rep529 element.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.04 MB.

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We declare that we have no other conflict of interest.

REFERENCES


