Detection of Toxoplasma gondii in aqueous humour by the polymerase chain reaction

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Abstract
Ocular toxoplasmosis is the most frequent infectious cause of choriotinal inflammation in immunocompetent patients. Nowadays, the biological diagnosis of ocular toxoplasmosis requires serological tests and anterior chamber puncture to detect the local production of specific antibodies. A new technique is described to detect Toxoplasma in aqueous humour by a polymerase chain reaction in which the target is a specific ribosomal DNA segment. Sixty eight patients (71 eyes) were included; 59 (83%) eyes were suspected of having ocular toxoplasmosis. Of these 59 eyes, 15 (25-4%) had characteristic fundus lesions with obvious intraocular inflammation signs and 44 (75%) had retinal scar of ocular toxoplasmosis without clinically detectable inflammation. Twelve (17%) eyes had uveitis of non-Toxoplasma origin and constituted the control group. The parasite was present in aqueous humour in 20 (33-8%) cases. No false positives were detected. The sensitivity of the test is reduced by the low numbers of the sample. The combination of this technique with Witmer-Desmonts coefficient increases the probability of making a biological diagnosis of ocular toxoplasmosis. The physiopathological value of this technique is emphasised and the presence of tachyzoites in the anterior chamber is suggested. This should be a very promising technique for the diagnosis of ocular toxoplasmosis.

Subjects and methods
This study was approved by the ethical committee of Bordeaux II University.

Subjects
Sixty eight patients (71 eyes) were selected, 32 (47%) men and 36 (53%) women. The mean age was 23-48 (range 3-69).

Patients were divided into three groups:
Group I. Forty four (62%) patients with retinal scar and who had an ACP in the past with a Witmer-Desmonts coefficient ≥ 4.
Group II. Fifteen (21-1%) patients with characteristic fundus lesions and intraocular inflammation signs indicating ocular toxoplasmosis in acute phase.
Group III. Twelve (16-9%) patients with posterior and/or anterior uveitis without clinical signs of a toxoplasomic origin, constituting the control group.

After a local anaesthetic or general anaesthesia with fluorotane for young children, an ACP was performed by taking 0-2 ml of aqueous humour after a limbic paraconitessen with microflex infusion gauge 27 needle. At the same time, a serological test determined the Witmer-Desmonts coefficient.

Methods
The quantity of Toxoplasma antibodies in the serum and aqueous humour was evaluated by using the indirect fluorescent antibody test. Immunological load was the ratio between the anti-Toxoplasma antibody titre (IU) and the IgG concentration (g/l). The Witmer-Desmonts coefficient was calculated by comparing the concentration of anti-Toxoplasma antibodies divided by the concentration of γ globulins in the aqueous humour to that in the serum. The coefficient was considered as positive when ≥ 4.

Toxoplasma DNA in aqueous humour was detected by a PCR assay, in which the target was part of a ribosomal DNA (rDNA) repetitive gene, as described. An 88 base pair segment was amplified with the synthetic oligonucleotide.
primers 5’ CCTTGCCCGATAGGTCTAGG and 5’ GGCATCTCTGTTGAAAGT. DNA from aqueous humour was obtained, after centrifugation, by a proteinase K-sodium dodecyl sulphate soft procedure according to Jeffreys et al. Briefly, the pellets were digested for 1 hour at 37°C in 1·5 μl of a buffer containing 5 mM TRIS-HCl (pH 7.5), 3·4 mM sodium dodecyl sulphate (0·01%), 40 mM dithiothreitol, 50 μg/ml proteinase K, and 0·1 mM of each primer, followed by a heating step of 10 minutes at 95°C.

After exposure to 95°C temperature for 10 minutes, DNA was amplified in a 50 μl reaction mixture containing 10 mM TRIS-HCl (pH 8·5), 16 mM (NH_4)_2SO_4, 2·5 mM TRIS-MgCl_2, 10 mM 2-mercaptoethanol, 1 mg/ml bovine serum albumin, 200 μM of each of all four deoxyribonucleoside triphosphates, 1 μM each of the two oligonucleotide primers and 1 unit of Taq polymerase (Cetus). Amplification in an automated thermal cycler (Perkin Elmer Cetus) consisted of 35 cycles (denaturation step 95°C for 10 seconds, annealing step 64°C for 30 seconds and synthesis step 70°C for 1 minute). 10 μl of each amplification reaction was analysed on 10% polyacrylamide gel and the DNA fragments were visualised by staining with ethidium bromide (Fig 1).

Results (Table 1)

<table>
<thead>
<tr>
<th>Biological tests</th>
<th>Groups</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>WDCandPCR+</td>
<td>5</td>
</tr>
<tr>
<td>WDCandPCR-</td>
<td>12</td>
</tr>
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<td>17</td>
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<tr>
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</tr>
<tr>
<td>Total</td>
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WDC = Witmer-Desmonts coefficient.
PCR = polymer chain reaction.

performed on purified *Toxoplasma gondii* DNA, less than 50 fg could be detected directly, which corresponds to the quantity of DNA in one parasite. Using hybridisation with an internal labelled probe after Southern blot analysis, it was possible to detect DNA corresponding to a single copy gene (1 fg).

CLINICAL SPECIMENS

**Group I**
The Witmer-Desmonts coefficient was ≥4 in 22 (50%) of cases. *Toxoplasma* DNA was detected in 15 (34%) cases; of these 15 cases, the Witmer-Desmonts coefficient was >4 in five (11·3%) and <4 in 10 (22·7%) respectively. Of the 12 (27·2%) remaining patients, the Witmer-Desmonts coefficients were <4 and no *Toxoplasma* DNA was detected.

**Group II**
The Witmer-Desmonts coefficient was ≥4 in five (33·3%) cases. *Toxoplasma* DNA was detected in five (33·3%) cases, the Witmer-Desmonts coefficient was >4 in one (6·6%) and <4 in four (26·6%) respectively. Of the six (40%) remaining patients, the Witmer-Desmonts coefficient was <4 and no *Toxoplasma* DNA was detected.

**Group III**
In all cases, the Witmer-Desmonts coefficient was <4 and the detection for *Toxoplasma* DNA was negative.

Discussion

There are several serological tests that demonstrate the presence of antibodies to *Toxoplasma*. However, because the prevalence of antibodies to *Toxoplasma* is high in certain communities, and since high titre antibodies may persist for years in otherwise healthy individuals, interpretation of these tests is often difficult. In countries such as France, 90% of adults have been infected with toxoplasmosis by the fourth decade. If the retinal lesion is atypical and the serological test is positive, diagnosis of *Toxoplasma* chorioretinitis is only presumptive because of the prevalence of antibodies in the population. In 1966 Desmonts described a method for comparing the ratio of anti-*Toxoplasma* antibodies in aqueous humour with those in serum. A coefficient of 4 or above is consistent with the intraocular production of specific anti-*Toxoplasma* antibodies, and suggests active ocular toxoplasmosis. We believe that the detection of *Toxoplasma* with PCR presents two main advantages: diagnostic and physiopathological.

In our series no false positive was noted (which confirms a high specificity), and with this technique, only one fragment of *Toxoplasma* DNA needed to be amplified for detection. The sensitivity of the test is, however, reduced by the low volume of the sample (0·2 ml) which may mean that a tachyzoite goes undetected while being present in the anterior chamber. When the...
Detection of *Toxoplasma gondii* DNA is positive and the Witmer-Desmonts coefficient negative, this could indicate that:

1. The activity of the ocular toxoplasmosis has been blocked by the local immunological response during the months before the ACP;
2. Some free organisms (tachyzoites) could be present in the anterior chamber without a significantly clinical inflammatory response;
3. The ACP is performed too early, before the Witmer-Desmonts coefficient becomes positive.

The detection of *Toxoplasma* DNA combined with the determination of the Witmer-Desmonts coefficient improves the probability of diagnosing ocular toxoplasmosis with biological tests. Indeed, if one compares the cases where the Witmer-Desmonts coefficient is >4 with those where the detection of *Toxoplasma* is positive, the biological diagnosis was made respectively in 50% and 34% of cases for group I, in 33-3% of cases for group II, and in 47-7% and 34% of cases for both groups. If the cases are added where the Witmer-Desmonts coefficient was positive and those where *Toxoplasma* DNA detection was also positive, the biological diagnosis was made in 72-7% of cases for group I, in 60% of cases for group II, and in 69-5% of cases for both groups.

Brezin *et al.* analysed samples of aqueous humour obtained from 17 patients with clinical symptoms consistent with ocular toxoplasmosis. Using PCR they detected *Toxoplasma gondii* DNA in the aqueous humour of three patients during two independent amplifications of the B1 gene fragment.

The various reports on the ultrastructural aspects of experimental ocular toxoplasmosis show the following:

1. Intracellular cysts are localised in the inner retina and particularly in the ganglion cell layer;
2. Organisms remain free in the intercellular medium for relatively short periods;
3. The inflammatory response concerns the choroid, the retina, and the vitreous;
4. In immunocompromised hosts, *Toxoplasma gondii* may infect the iris, the choroid, and the vitreous, tissues that are not usually infected in the immunocompetent host. Using the highly sensitive PCR, the presence of *Toxoplasma gondii* in the aqueous humour may be suggested.

**Conclusion**

Direct detection of *Toxoplasma* in aqueous humour can be a decisive complement to the diagnosis of ocular toxoplasmosis in certain atypical or clinically unclear cases. We report a PCR in which the target is a rDNA fragment.

In this work, we show the following:

1. The high specificity of this technique (no false positive).
2. The limited sensitivity of this test; this is because of the low volume of the sample which may preclude detection of *Toxoplasma*.
3. An increased probability of making a biological diagnosis by means of the combination of this technique and the Witmer-Desmonts coefficient.
4. The possible or probable presence of free parasites (tachyzoites) in the anterior chamber.

This technique promises to be effective for the diagnosis of ocular toxoplasmosis especially when combined with the Witmer-Desmonts coefficient.