Protocol for the Use of Polymerase Chain Reaction in the Detection of Intraocular Large B-Cell Lymphoma in Ocular Samples

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To determine the usefulness of polymerase chain reaction (PCR) analyses in the diagnosis of lymphoid infiltrate cells in ocular samples, PCR was performed using oligonucleotide primers specific for immunoglobulin heavy chain rearrangement at framework 2, framework 3, and t(14;18) translocation of the bcl-2 gene. These were used to successfully generate amplicons of 220 to 230 bp, 110 to 120 bp, and 175 to 200 bp, respectively. After PCR amplification, primers directed against the t(14;18) detected 10 pg of B-cell lymphoma DNA. PCR against Fr2 and Fr3 IgH rearrangement detected 10 fg and 10 pg in the seminested PCR, respectively. Conventional pathological methods were highly accurate at establishing the correct final diagnosis in formalin-fixed, paraffin-embedded samples but were much less sensitive and predictive in cytological specimens of intraocular fluid. A combination of the three PCR reactions was an equally successful diagnostic approach on paraffin-embedded samples, whereas single PCR reactions did not significantly improve diagnosis over histopathological diagnostic techniques. Thus, a combination of PCR reactions is useful in the detection of B-cell monoclonality, aids the differentiation between lymphomatous and inflammatory infiltrates, and is more powerful as a diagnostic method than single PCR or conventional cytopathology for lymphoid infiltrates in ocular fluid aspirates. (J Mol Diagn 2007, 9:113–121; DOI: 10.2353/jmoldx.2007.050121)

The incidence of primary ocular-central nervous system lymphoma is rising in the aging population, and the difficulty in establishing the diagnosis in these sites delays the onset of treatment, leading to increased morbidity and mortality.^{1,2} In the eye, lymphoma masquerades as sight-threatening intraocular inflammation that can have a variety of causes in all age groups.^{3,4} Treatments given to these patients, with high-dose corticosteroids and other

immunosuppressive agents, have considerable morbidity in the elderly patient and are unhelpful in treating the underlying problem, possibly masking it.²

The rate of diagnosis using conventional cytological and histological methods on samples of intraocular fluids is low, with the result that intraocular large B-cell lymphoma remains extremely difficult to diagnose and/or exclude.^{5,6} Association of raised interleukin-10 and the presence of primary ocular-central nervous system lymphoma in the vitreous have been reported as pointing toward a diagnosis of lymphoma.^{6,7} However, the diagnosis of malignancy requires certainty, and other inflammatory conditions may cause cytokines to be elevated.⁸

Immunoglobulin receptor genes exist as discontinuous segments of DNA that rearrange during the course of normal B-cell development.^{9–11} The variable region of the rearranged immunoglobulin heavy chain (IgH) contains three hypervariable complementary determining regions (CDRI, CDRII, CDRIII) and three relatively conserved framework regions (Fr1, Fr2, Fr3). Although not an absolute, lymphomatous populations of B cells are monoclonal expansions, whereas reactive/inflammatory processes, in which several antigenic epitopes stimulate the response, tend to be polyclonal or oligoclonal at least. Assessment of clonality is, therefore, a useful measure for the detection of lymphoma. Monoclonality in a B-cell population can be deduced by the production of a single discrete amplicon after polymerase chain reaction (PCR) of the immunoglobulin heavy chain gene variable region. The amplification product from a polyclonal population will result from a large number of rearranged Ig genes, which will give rise to amplicons of varying length, resulting in a smear or a broad band, when viewed by gel electrophoresis.¹²

Many non-Hodgkin's lymphomas show a balanced translocation of t(14;18). This translocation causes a fusion of *bcl-2* gene on chromosome 18 with the J region of the Ig gene on chromosome 14. In ~60% of such lymphomas, the breakpoint occurs in exon 3 of *bcl-2*, which is referred to as a major breakpoint region (MBR),^{13,14}

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and in 25% of cases, the breakpoint occurs in the minor cluster region.¹⁵ The MBR translocation can be detected by PCR using a 5' primer homologous to the *bcl-2* gene on chromosome 18 and a 3' primer complementary to the consensus sequence in the J_H region on chromosome 14.¹³

In recent years, molecular pathology techniques have enhanced the diagnostic approach to malignant lymphoma. PCR analysis has shown great potential in detecting and amplifying low gene copy numbers within DNA derived from clinical samples. It holds great potential in allowing the identification of a low number of cells in small clinical samples.¹⁶ Shen and colleagues^{17,18} used microdissection and PCR to detect gene rearrangement in the Fr3 IgH variable region and *bcl-2* MBR in primary central nervous system lymphoma.¹⁹ Other groups have also used *bcl-2* minor cluster region and IgH rearrangement to detect B-cell lymphoma in vitreous specimens.^{20,21}

In this study, we have assessed PCR-based techniques to derive a reliable and effective protocol for the analysis of immunoglobulin heavy chain rearrangement and *bcl-2* t(14;18) translocation for detection of monoclonality in ocular samples potentially containing lymphoma cells. We have assessed our methods in both formalin-fixed, paraffin-embedded samples and freshly frozen samples. The aim was to find a method that would increase the number of intraocular samples from which a confident diagnosis of lymphoma can be made or excluded, overcome the uncertainty of cytopathological diagnosis, and reduce the time taken to diagnosis.^{22–25}

Materials and Methods

Samples and Cell Cultures

Twenty-eight clinical ocular samples taken for diagnostic purposes at Moorfields Eye Hospital (London, UK) were included in the study. These patients all had intraocular cellular infiltrates in which intraocular lymphoma needed to be excluded. All underwent conventional histological or cytological examination for detection of lymphoma cells, and the final clinical diagnosis is now known in all cases. Of the 28 samples studied, 15 samples were formalin-fixed and paraffin-embedded, with 13 of these diagnosed as lymphoma and two as inflammatory. The remaining 13 samples were unfixed vitreous samples, three of which were considered to be lymphoma and 10 inflammatory. Normal vitreous was obtained from three patients undergoing vitrectomy for macular hole surgery. The study was approved by the local ethics committee. The B-cell lymphoma cell lines DOHH-2 and RAJI were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and European Collection of Cell Cultures, Salisbury, Wiltshire, UK, respectively.

Collection of Vitreous Fluid

Intraocular fluid samples are routinely taken for diagnostic purposes from patients with suspected intraocular

lymphoma in our practice. To take these samples, the extraocular environment is first disinfected with 5% povidone iodine solution. Vitreous samples (200 to 400 μ l) are taken using either a needle as an aspiration biopsy or using a vitrector during vitrectomy. These samples are immediately transported to the pathology laboratory for urgent processing. In this study, for standard cytological analysis, samples were processed to produce direct smears and/or cytospin preparations and/or formalin-fixed and cytoblocked before staining with either hematoxylin and eosin and modified Giemsa stain or immunohistochemistry using a panel of CD markers according to the preference of the duty pathologist.²⁵ After this routine diagnostic approach, residual fluid samples, where available, were snap frozen and stored at -80°C pending further molecular analysis.

Positive and Negative Controls

Genomic DNA from the B-cell lymphoma cell lines DOHH-2 and RAJI were used as positive controls. Sterile water was used as a PCR-negative control. Normal vitreous samples from patients undergoing macular hole surgery were used as negative control samples and were snap-frozen before extraction of DNA using methods identical to those used with frozen samples of vitreous suspected of containing lymphoma.

Extraction of DNA from Paraffin-Embedded and Frozen Samples

Briefly, 15- μ m sections of paraffin-embedded retina were cut, transferred to a 1.5-ml microcentrifuge tube, and deparaffinized by one xylene extraction. Xylene (1.2 ml) was added, and the sample was vortexed and centrifuged at room temperature for 5 minutes to pellet the tissue. Supernatant was removed with a pipette and then washed twice with 1.2 ml of 100% ethanol to remove xylene. After evaporating the ethanol from the tissue pellet, DNA was extracted using a Qiaamp DNA extraction mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The Qiaamp DNA mini kit was also used to extract DNA from frozen DOHH-2 and RAJI cell lines and frozen vitreous samples of patients. All extracted DNA was diluted to a concentration of 10 ng/ μ l and stored at -20°C in sterile Tris-ethylenediaminetetraacetic acid (TE) buffer (10 mmol/L Tris and 1 mmol/L ethylenediaminetetraacetic, pH 8.0). One- μ l samples of DNA were used per 50 μ l PCR reaction. Five- μ l aliquots were heated to 95°C for 20 minutes before undergoing PCR amplification using primers directed against Fr2, Fr3, and *bcl-2* translocation.

PCR

Details of the primers used for the main part of this study are shown in Tables 1 and 2.^{12,21,25} All primers used were synthesized by Sigma-Genosys Ltd. (The Woodlands, TX). First- and second-round reactions contained 200

Table 1. IgH Rearrangement Gene Primer Sequence

Oligonucleotide primer	Sequence
FR2 sense strand primer	5'-TGGATCCGCCAGGCTTCAGG-3'
FR3 sense strand primer	5'-ACACGGCTGTGTGTATTACTGTG-3'
LJH outer primer (nonsense strand)	5'-ACCTGAGGAGACGGTGACC-3'
VLJH inner primer (nonsense strand)	5'-GTGACCAGGGTACCTTGGCCCCAG-3'

μ mol/L of each primer, varying concentrations of $MgCl_2$ depending on the primer pairs used in the reaction, 200 μ mol/L dNTP, 2.5 U of *Taq*DNA polymerase, and buffer (Qiagen HotStar Master Mix kit) in a 50- μ L reaction. PCR cycling was performed at 95°C for 15 minutes for one cycle, followed by 35 cycles at 95°C for 30 seconds, annealing temperature as stated for 30 seconds, and extension at 72°C for 30 seconds. The final cycle was followed by a 10-minute extension phase at 72°C.

Electrophoresis and Imaging

Ten- μ L amplification products were visualized under UV illumination after electrophoresis on 8% polyacrylamide gel electrophoresis gels (PAGE)/Tris borate-ethylenediamine tetraacetic acid (TBE) gels and staining with SYBR Gold and ethidium bromide. A molecular weight marker was included in each run (50-bp ladder; Promega UK Ltd., Southampton, UK).

DNA Sequencing

Before sequencing of PCR products, amplified DNA from each PCR was purified using the QIAquick PCR purification kit (Qiagen). PCR products were excised from 3% low melting point agarose-TAE gel, solubilized, and recovered into solution according to the manufacturer's instruction. PCR fragments were directly cycle-sequenced in both directions (using primers Fr2 + LJH, Fr2 + VLJH, Fr3 + LJH, Fr3 + VLJH, and MBR + LJH) on an ABI Prism automated DNA sequencer (model 377, version 2.1.1; Applied Biosystems, Foster City, CA).

Results

Amplification of Fr2, Fr3, and BCL2 MBR Translocation t(14;8) from Human B-Cell Lymphoma DNA

A single amplicon with a predicted size of 230 bp was amplified using the first-round Fr2/LJH primers using B-cell lymphoma DNA as template. This amplification was

capable of detecting 10 pg of B-cell lymphoma DNA after analysis on 8% SYBR Gold-stained 8% PAGE/TBE gels. Subsequent amplification with nested primers yielded a 220-bp target sequence that was able to detect 10 fg of target DNA (Figure 1, A and B; Table 3).

Amplification of B-cell lymphoma DNA using primers specific for Fr3 region (Fr3/LJH) showed similar results with first-round amplification detecting a single amplicon of 120 bp to a sensitivity of 100 pg. Nested amplification yielded a single amplicon of 110 bp and showed a 10-fold increase in sensitivity (Figure 1, C and D; Table 3). A 200-bp sequence from the MBR was used as the target sequence for amplification to detect the BCL2 MBR t(14;18) translocation. Amplification of the t(14;18) translocation gene from B-cell lymphoma allowed detection of 100 pg of DNA. Subsequent nested amplification yielded a 175-bp product with a 10-fold increase in sensitivity (Figure 1, E and F; Table 3).

Negative controls were included in each protocol, and the first-round negatives were included as test samples in the seminested PCR reaction. Negative control samples from both rounds of PCR were consistently negative after two rounds of amplification. All first-round and nested PCR products were cycle-sequenced and confirmed to be identical with the published sequences. Identical amplifications demonstrated that the presence of 20% normal human vitreous did not inhibit PCR amplification of these target sequences using the above primer sets. No amplification product was obtained with genomic DNA isolated from *Aspergillus fumigatus*, *Fusarium solani*, human vitreous, human blood, or 13 species of bacteria. A band was seen with the positive lymphoma controls and not with any negative controls.

Patient Samples

Twenty-eight patient samples underwent analysis using the Fr2, Fr3, and *Bcl-2* t(14;18) oligonucleotide primers. (Figures 2, 3, and 4) These samples and the results of conventional diagnostic methods and PCR analysis are summarized in Table 4. All 13 formalin-fixed, paraffin-embedded samples from patients with histopathologically proven large B-cell intraocular cell lymphoma were

Table 2. Bcl-2 t(14;18) Translocation Gene Primer Sequences

Oligonucleotide primer	Sequence
Sense strand primer MBR(a)	5'-TTAGAGAGTTGCTTTACGTGGCCT-3'
Inner sense strand primer MBR(b)	5'-TTTGACCTTTAGAGAGTTGC-3'
Nonsense outer strand primer LJH	5'-ACCTGAGGAGACGGTGACC-3'
Nonsense inner strand primer VLJH	5'-GTGACCAGGGTACCTTGGCCCCAG-3'

Figure 1. Amplification of Fr2, Fr3, and Bcl-2 t(14;18) PCR product from purified genomic DNA. **A:** Varying quantities of DOHH B-cell lymphoma genomic DNA were subjected to PCR amplification using Fr2 primers, which detected 10 pg of B-cell lymphoma DNA. PCR products (10 μ l) were resolved on 8% PAGE/TBE gels and visualized after SYBR Gold staining under UV illumination. **B:** Seminested amplification of Fr2 PCR product generated from purified genomic DNA detected 10 fg of B-cell DNA, using seminested Fr2 primers. PCR products (10 μ l) were resolved on 8% PAGE/TBE gels and visualized after ethidium bromide staining under UV illumination. **C:** Varying quantities of RAJI B-cell lymphoma genomic DNA were subjected to PCR amplification using Fr3 primers, which detected 100 pg of B-cell lymphoma DNA. PCR products (10 μ l) were resolved on 8% PAGE/TBE gels and visualized after SYBR Gold staining under UV illumination. **D:** Seminested amplification of Fr3 PCR product generated from purified genomic DNA detected 10 pg of B-cell DNA, using seminested primers Fr3 primers. PCR products (10 μ l) were resolved on 8% PAGE/TBE gels and visualized after ethidium bromide staining under UV illumination. **E:** Varying quantities of DOHH B-cell lymphoma genomic DNA were subjected to PCR amplification using specific t(14;18) primers, which detected 100 pg of B-cell lymphoma DNA. PCR products (10 μ l) were resolved on 8% PAGE/TBE gels and visualized after SYBR-Gold staining under UV illumination. **F:** Nested amplification of a PCR product from the Bcl-2 translocation t(14;18) gene from purified genomic DNA detected 10 pg of B-cell lymphoma DNA. PCR products (10 μ l) were resolved on 8% PAGE/TBE gels and visualized after ethidium bromide staining under UV illumination.

appropriately positive on PCR analysis. The two additional formalin-fixed, paraffin-embedded samples that were diagnosed as inflammatory on histopathological analysis were negative by PCR. These patients were diagnosed as chronic immune-mediated uveitis, and their clinical details show that throughout at least 3 years of follow-up there has been no evidence of intraocular lymphoma.

The 13 fresh frozen vitreous samples consisted of three in which cytological assessment had indicated lymphoma and 10 in which inflammation was the cytological opinion after conventional examinations. All three of the lymphoma samples were positive by PCR. However, only 6 of the 10 inflammatory samples were

negative—four gave a positive result, suggesting the presence of lymphoma. In each of the four cases in which the cytopathology was negative for lymphoma and the PCR-positive, the cytopathology revealed adequate samples with reasonable cellularity. The cellular content of the aspirates was mixed and included lymphocytes of varying sizes, macrophages, and in some cases neutrophil polymorphs. In most cases, the lymphocyte populations were demonstrated as a mixture of T and B cells on immunocytochemistry with anti-CD3 and -CD20 antibodies. In no case was it possible to unequivocally diagnose the presence of B-cell intraocular lymphoma based on the appearances presented. All four were subsequently diagnosed as intraocular lymphoma af-

Table 3. Sensitivity of PCR Amplification

Amplicon	Annealing	Mg ²⁺ concentration (mmol/L)	Sensitivity (DNA)	Size (bp)
Forward Fr2 IgGH	61°C	1.5	10 pg	230
Seminested Fr2 IgGH	62°C	2.0	10 fg	220
Forward Fr3 IgGH	58°C	2.5	100 pg	120
Seminested Fr3 IgGH	58°C	2.5	10 pg	110
Forward BCL2 MBR	62°C	3.0	100 pg	200
Nested BCL2 MBR	62°C	1.5	1 pg	175

Figure 2. Seminested amplification of Fr2 PCR product generated from purified genomic DNA derived from patient samples. Positive control derived from DOHH genomic DNA. All PCR products (10 μ l) were resolved on 8% PAGE/TBE gels and visualized after ethidium bromide staining under UV.

ter follow-up and further biopsy, cerebrospinal fluid analysis, or systemic biopsy.

In the four cases in which the cytopathology was negative for lymphoma and the PCR-positive, the clinical features varied. The first was a 65-year-old female with bilateral uveitis that had previously had a treated systemic B-cell lymphoma. The first vitreous tap was nondiagnostic. The second tap was regarded as showing an inflammatory reaction and was not diagnostic of lymphoma. The patient then suffered an overt recurrence of her lymphoma in other nonocular sites, was treated with systemic chemotherapy, and experienced a resolution of the uveitis as a consequence.

The second case was a 69-year-old female who had previously been treated for intraocular lymphoma, resulting in remission before presenting with a 4-month history of worsening pan-uveitis. The vitreous smear was interpreted as showing a reactive mixed lymphocytic picture that was not diagnostic of lymphoma. Follow up demonstrated worsening of her condition with lymphoma demonstrated in her cerebrospinal fluid.

The third case was an 84-year-old male who had a nonsteroid responsive uveitis that seemed to be predominantly attributable to T cells and macrophages on vitreous cytology. However, cerebrospinal fluid cytology

demonstrated the presence of a B-cell lymphoma, and the patient subsequently died.

The fourth case was a 60-year-old female with a previous history of metastatic breast carcinoma who presented with bilateral pan-uveitis. The cytological picture in the vitreous was interpreted as showing a reactive pattern. Further investigation by an oncologist resulted in the diagnosis of central nervous system B-cell lymphoma, positive by cerebrospinal fluid cytopathology. The patient was treated with chemotherapy and radiotherapy.

In these cases, unequivocal tissue-based diagnoses of lymphoma were subsequently obtained in nonocular sites after negative cytopathological assessments of the intraocular disease but positive PCR results. Irrefutable evidence of intraocular lymphoma was never provided in these cases, although the subsequent clinicopathological picture was highly suggestive of intraocular involvement by lymphoma.

Agreement between morphological histo/cytopathologically based and PCR-based diagnoses were assessed using κ statistics. For the whole group of patient samples, κ is 0.6 (95% CI, 0.3 to 0.9; good agreement). κ is 1 when comparing the methods on the formalin-fixed, paraffin-embedded samples because of the perfect concordance between the two methods. However, for specimens of vitreous intended for cytological assessment, κ is 0.4 (95% CI, -0.1 to 0.7; only fair agreement).

For each method used, the sensitivity, specificity, positive predictive value, and negative predictive value were calculated with reference to the final clinicopathological diagnosis (Table 5). For specimens processed by formalin fixation and paraffin-embedding for histology, histopathology achieved a perfect diagnostic result predictive of the final clinicopathological diagnosis in each case and was equally matched by a combination of three PCR methods or by a combination of PCR reactions for FR2 and FR3. However, for specimens that required a cytopathology approach to morphological diagnosis, the sensitivity was only 0.33 and the negative predictive value was 0.5, although specificity and positive predictive value were 1.0. In contrast, by using a PCR approach, sensitivity and specificity of 1.0 could be achieved in these cases using the combination of three PCR analyses. Sensitivity was reduced using only a single PCR for FR2, FR3, or MBR but was increased by combining two of these PCR reactions in the

Figure 3. Seminested amplification of Fr3 PCR product generated from purified genomic DNA derived from patient samples. Positive control derived from RAJI genomic DNA. All PCR products (10 μ l) were resolved on 8% PAGE/TBE gels and visualized after ethidium bromide staining under UV.

Figure 4. Nested amplification of PCR product from the Bcl-2 translocation t(14;18) gene from purified genomic DNA derived from patient samples. Positive control derived from DOHH genomic DNA. All PCR products (10 μ l) were resolved on 8% PAGE/TBE gels and visualized after ethidium bromide staining under UV.

Table 4. Summary of the Clinical Cases Studied with a Comparison of Histo-/Cytopathology Opinions and PCR Results as a Predictor of Final Clinicopathological Diagnosis

Patient no.	Sample type	Processing method of sample	Histo-/Cytopathology opinion	PCR results		
				Fr2	Fr3	MBR t(14;18)
1	Enucleation	FFPE	B-cell lymphoma	+	+	—
2	Choroidal biopsy	FFPE	B-cell lymphoma	+	+	—
3	Enucleation	FFPE	B-cell lymphoma	+	+	—
4	Choroidal biopsy	FFPE	B-cell lymphoma	+	+	+
5	Vitreous aspirate	Cytoblock	B-cell lymphoma	+	—	—
6	Retinal biopsy	FFPE	B-cell lymphoma	—	+	+
7	Uveal biopsy	FFPE	Chronic inflammation	—	—	—
8	Retinal biopsy	FFPE	B-cell lymphoma	+	+	+
9	Uveal biopsy	FFPE	B-cell lymphoma	—	+	—
10	Enucleation	FFPE	Chronic inflammation	—	—	—
11	Enucleation	FFPE	B-cell lymphoma	—	+	—
12	Retinal biopsy	FFPE	B-cell lymphoma	—	+	—
13	Vitreous aspirate	Fresh frozen	B-cell lymphoma	—	+	—
14	Uveal biopsy	FFPE	B-cell lymphoma	—	+	—
15	Enucleation	FFPE	B-cell lymphoma	+	+	—
16	Vitreous aspirate	Fresh frozen	Chronic inflammation	—	—	—
17	Vitreous aspirate	Fresh frozen	Chronic inflammation	—	—	—
18	<i>Vitreous aspirate</i>	<i>Fresh frozen</i>	<i>Chronic inflammation</i>	+	—	—
19	<i>Vitreous aspirate</i>	<i>Fresh frozen</i>	<i>Chronic inflammation</i>	—	+	—
20	<i>Vitreous aspirate</i>	<i>Fresh frozen</i>	<i>Chronic inflammation</i>	—	+	—
21	Vitreous aspirate	Fresh frozen	B-cell lymphoma	+	—	—
22	Vitreous aspirate	Fresh frozen	Chronic inflammation	—	—	—
23	<i>Vitreous aspirate</i>	<i>Fresh frozen</i>	<i>Chronic inflammation</i>	—	+	—
24	Vitreous aspirate	Fresh frozen	Chronic inflammation	—	—	—
25	Vitreous aspirate	Fresh frozen	B-cell lymphoma	+	+	—
26	Enucleation	FFPE	B-cell lymphoma	+	—	+
27	Vitreous aspirate	Fresh frozen	Chronic inflammation	—	—	—
28	Vitreous aspirate	Fresh frozen	Chronic inflammation	—	—	—

FFPE, formalin-fixed, paraffin-embedded.

Italicized cells, samples discordant between cytopathology and PCR results; bold, samples in which PCR suggested a diagnosis of lymphoma in spite of a nondiagnostic cytopathology assessment.

(Table continues)

diagnostic approach, with a combination of FR2 and FR3 being the best tandem PCR combination.

Discussion

The determination of clonality is extremely helpful in the analysis of lymphoproliferative disorders. Benign lymphoproliferations are typically polyclonal or oligoclonal, whereas most malignant lymphomas in immunocompetent individuals are monoclonal. Speed, ease, and the ability to amplify small amounts of DNA are virtues associated with the PCR technique. In this study, we applied molecular biological techniques to the detection of large B-cell intraocular lymphoma in ocular samples. Immunoglobulin heavy chain (IgH) rearrangements in aberrant second (Fr2) and third (Fr3) framework regions^{11,23,25–28} and the *bcl-2* translocation t(14;18)^{13–15,22} were successfully detected using PCR. These are fast, simple, and economical techniques that are rapidly becoming more commonly used for the routine diagnosis of B-cell monoclonality.

The configuration of an IgH gene rearrangement is unique to an individual B cell and its progeny and can, therefore, be used as a marker. The PCR methods described have used a variety of consensus primers that

hybridize with conserved sequences present in most of the variable and joining regions of the IgH gene and the translocation of the *bcl-2* gene t(14;18).^{12,22,27} The size of amplified products is easily analyzed by gel electrophoresis, with monoclonal populations of B cells giving rise to one or two dominant bands, whereas polyclonal lesions give a range of product sizes, which appear as a smear or ladder of bands in the gel.

Amplification of the Fr3 of the IgH gene region and *bcl-2* translocation has been previously applied to ocular tissues,^{7,17,18,20,21} the former as a seminested reaction and the latter as a nested PCR reaction. The method to detect monoclonality using the Fr3 primers alone have the disadvantages that 20 to 30% of B-cell neoplasms are not detected and that the efficiency of amplification is highly variable.¹² Several other studies have suggested inefficient recognition by the consensus of certain VH or JH families, incorrect or incomplete IgH gene rearrangements, the presence of abundant polyclonal B cells that might mask the monoclonal population, and chromosomal translocation rendering one allele unsuitable for VDJ amplification.²⁹ A negative result does not completely exclude the presence of a monoclonal population and monoclonality does not necessarily denote neoplasia. False-positive reactions can be seen in microdissected specimens because of

Table 4. *Continued*

Final clinicopathological diagnosis	Pathology predictive of diagnosis? (benign versus malignant)	PCR predictive of diagnosis? (benign versus malignant)	Pathology predictive of outcome?	PCR predictive of outcome?
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
Chronic inflammation	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
Chronic inflammation	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
Chronic inflammation	Y	Y	Y	Y
Chronic inflammation	Y	Y	Y	Y
<i>B-cell lymphoma</i>	<i>N</i>	Y	<i>N</i>	Y
<i>B-cell lymphoma</i>	<i>N</i>	Y	<i>N</i>	Y
<i>B-cell lymphoma</i>	<i>N</i>	Y	<i>N</i>	Y
B-cell lymphoma	Y	Y	Y	Y
Chronic inflammation	Y	Y	Y	Y
<i>B-cell lymphoma</i>	<i>N</i>	Y	<i>N</i>	Y
Chronic inflammation	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
B-cell lymphoma	Y	Y	Y	Y
Chronic inflammation	Y	Y	Y	Y
CMV	Y	Y	Y	Y

Table 5. A Comparison of the Sensitivity, Specificity, and Positive and Negative Predictive Values of Histo-/Cytopathology-Based Diagnoses and PCR-Based Diagnoses Relative to the Final Clinicopathological Diagnosis

Diagnostic method	Sample group	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Histo-cytopathology	Entire cohort	0.73	1	1	0.57
	FFPE cohort only	1	1	1	1
	Vitreous cytology cohort only	0.33	1	1	0.5
Combination of three PCR results	Entire cohort	1	1	1	1
	FFPE cohort only	1	1	1	1
	Fresh frozen vitreous cohort only	1	1	1	1
FR2 PCR	Entire cohort	0.54	1	1	0.44
	FFPE cohort only	0.62	1	1	0.29
	Fresh frozen vitreous cohort only	0.44	1	1	0.54
FR3 PCR	Entire cohort	0.73	1	1	0.57
	FFPE cohort only	0.85	1	1	0.5
	Fresh frozen vitreous cohort only	0.56	1	1	0.6
MBR PCR	Entire cohort	0.23	1	1	0.32
	FFPE cohort only	0.31	1	1	0.18
	Fresh frozen vitreous cohort only	0.11	1	1	0.43
FR2 + FR3 PCR	Entire cohort	0.95	1	1	0.89
	FFPE cohort only	1	1	1	1
	<i>Fresh frozen vitreous cohort only</i>	<i>0.89</i>	<i>1</i>	<i>1</i>	<i>0.86</i>
FR2 + MBR PCR	Entire cohort	0.64	1	1	0.50
	FFPE cohort only	0.69	1	1	0.33
	Fresh frozen vitreous cohort only	0.56	1	1	0.6
FR3 + MBR PCR	Entire cohort	0.82	1	1	0.67
	FFPE cohort only	0.92	1	1	0.67
	Fresh frozen vitreous cohort only	0.67	1	1	0.67

Bold, ideal diagnostic tests in the defined cohorts; italics, next best choice diagnostic approach in the defined cohorts.

analysis performed on a small number of reactive lymphocytes sharing a clonal origin, low number of target sequences resulting in preferential amplification of certain VDJ configurations, or fragments of tissue containing local clusters of clonal or clonally related cells.¹⁹

The t(14;18) translocation is found in the majority of follicular and in some nonfollicular lymphomas. In more than half of the cases, the breakpoint on chromosome 18 falls within a 150-bp region (MBR). The chromosome 18 fragment always translocates to chromosome 14 at one of the six J segments of the immunoglobulin heavy chain locus. The PCR methods previously described amplify a small translocation-specific fragment.^{13–15} In theory, this fragment, encompassing the chromosomal juncture and is unique to the malignant cells, which are the only translocation-bearing cells. However, it has also been shown that few cells with this translocation are present in a significant proportion of reactive lymphoid hyperplasia.

In this study, to overcome the drawbacks of any single PCR approach, we have used a panel of primers for the Fr2 and Fr3 of the IgH gene in a seminested reaction, together with a nested reaction for the *bcl-2* t(14;18) translocation, to increase the sensitivity and efficiency of detection of monoclonality of large B-cell intraocular lymphomas and decrease the false-negative cases. To ensure specificity of PCR reactions, because polyclonal cells may be present in ocular samples, we have opted for the highest annealing temperature possible without reducing product yield.

This study has demonstrated that a combination of all three PCR-based techniques achieved the correct diagnosis in 100% of the patients in our study cohort with a final clinicopathological diagnosis of intraocular lymphoma, whether the diagnostic sample had been processed for formalin fixation and paraffin-embedding or was an intraocular fluid sample more usually assessed by cytopathological methods. Where histopathological diagnosis is available on formalin-fixed, paraffin-embedded material, our data suggest that this approach remains the standard because it was capable of producing the correct diagnosis in all cases in this series and it is also additionally capable of providing information such as cell type or further classification of disease and of inferring a more accurate diagnosis in inflammatory cases, such as that of a specific intraocular infection. If discrepant results are obtained from tissue diagnosis, then a further search should be made with further repeat biopsies from the vitreous humor or other sites.^{30–32} However, if diagnostic doubt remains over lymphoma versus inflammation, we can confirm that DNA extracted from formalin-fixed, paraffin-embedded tissues can be amplified successfully, permitting the analysis of clonality in archival material.^{22,33–35} Standard DNA extraction methods are time consuming and involve multiple tube transfers in which cells and DNA may be lost. For the purpose of application in ocular samples, in which the sample volume is small, Qiagen DNA Qiaamp mini kit was used to reduce the potential loss of cells and DNA in transfer between tubes. This method not only reduced the complexity of DNA extraction (and thereby the time) but also achieved a high sensitivity, as judged by subsequent PCR.

The cytopathological diagnosis of aspirates of intraocular lymphoid infiltrates may be difficult by morphology alone, for a variety of reasons including artifacts as a result of cell fragility or the paucicellular nature of many aspirates, thereby delaying appropriate treatment.^{2,24,32,35} Diagnostic accuracy can be improved by using ancillary immunohistochemistry if sufficient material is available. For example, B-cell monoclonality may be inferred by demonstrating monotypic immunoglobulin (Ig) light chain expression using immunohistochemistry.^{31,36} However, this technique is not successful in all cases or in all laboratories and may be difficult to interpret—especially if only a minor lymphomatous component is present in the tissue. Frequently, it is difficult to interpret these samples because cellular details may be degenerate as a result of the fluid environment in which they are suspended, physical damage to the cells by surgical instruments,^{2,37,38} or because prior treatment with steroids has altered the cellular composition of the infiltrate.^{5,17,39} Furthermore, too few cells may be obtained, or a small population of tumor cells may be obscured by an abundance of reactive lymphocytes, macrophages, and other cells.⁴⁰ Multiple sampling procedures are often required to make a correct diagnosis.^{40–43}

In this series, cytopathological techniques were used in 13 cases of vitreous aspirates with limited success. Sensitivity of diagnosis by this method and the negative predictive value of the test were low, although specificity and positive predictive value were high. A PCR-based diagnosis using a single reaction for FR2, FR3, or MBR alone provided little benefit over traditional cytopathological diagnosis. However, in combination all three tests were able to correctly predict the final clinicopathological diagnosis in all vitreous samples in this series. Inclusion of the PCR reaction for the MBR provided relatively little to the diagnostic accuracy, and an excellent, although not perfect, diagnosis could be made by PCR in all but 1 of the 13 aspirates (1 in 28 cases of the entire sample cohort) if this test had been omitted. Therefore, our results suggest that a combination approach of PCR for FR2 and FR3, with or without MBR, could significantly improve the diagnostic accuracy in vitreous aspirates over that achieved by cytopathology.

Detection of monoclonality in lymphoid infiltrates is strongly suggestive of neoplastic disease. Therefore, if applied in cases of clinical suspicion and also with other diagnostic procedures, we believe that this technique is of value because of its speed, simplicity, sensitivity, and specificity. PCR using a panel of primers directed toward IgH rearrangement, Fr2, Fr3, and *bcl-2* t(14;18) regions as an adjunct to cytology, especially in uncertain cases, will be helpful in the diagnosis and management of ocular lymphomas.

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