Original Article

MYD88 L265P mutation in intraocular lymphoma: A potential diagnostic marker

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Purpose: Vitreoretinal lymphoma (VRL) is the most common intraocular lymphoma (IOL). This can be either primary or secondary to the central nervous system lymphoma. The diagnosis of primary intraocular lymphoma (PIOL) currently relies on clinical diagnosis and cytological analysis of the vitreous or subretinal biopsy. Although most cases are diagnosed without much issue, the limited amount of vitreous fluid, subjectivity in cytological reporting, and special expertise in ocular pathology make the diagnosis challenging. *MYD88* L265P mutation has been implicated to have diagnostic utility in PIOL. In this study, we screened consecutive vitreous biopsies for the presence of *MYD88* L265P mutation to understand its diagnostic utility compared to conventional cytological analysis. **Methods:** Cytological analysis and *MYD88* L265P mutation by PCR-based sequencing and restriction fragment length polymorphism (RFLP) were carried out on consecutive vitreous and subretinal biopsies collected from 21 patients. The diagnostic utility of the cytology and *MYD88* L265P mutation analysis were compared. **Results:** Out of the 21 patients, 15 had clinical suspicion of having PIOL. Out of these suspected cases of PIOL, nine were confirmed on follow-up, while six were diagnosed as other intraocular pathologies. Diagnostic utility of *MYD88* L265P mutation analysis revealed a sensitivity of 88.9%, specificity of 91.6%, positive and negative predictive value of 88.9% and 91.7%, respectively. Diagnostic accuracy of 90.5% was achieved with the mutation analysis that shows the superiority of *MYD88* in both ruling in and ruling out PIOL. The diagnostic utility of *MYD88* L265P mutation was superior to conventional cytological analysis. **Conclusion:** The analysis of *MYD88* L265P mutation is reliable and efficient in the diagnosis of PIOL.

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Primary intraocular lymphoma (PIOL) refers to a B cell non-Hodgkin's lymphoma of the retina and vitreous, sometimes with concomitant central nervous system (CNS) involvement. The diagnosis of PIOL is a team effort by the ophthalmologist, pathologist who uses both light microscopy to study the cell morphology and immunocytochemistry for CD20 to establish the diagnosis. Cytological observation of large, atypical lymphoid cells with increased nuclear/cytoplasmic ratio, basophilic cytoplasm, and irregular nuclei is seen in cases of PIOL. However, there are challenges in the diagnosis of PIOL because of the lower volumes of samples available in the form of vitreous aspirate/subretinal aspirate, prior treatment with steroids and lower representation of lymphoma cells amidst a mixture of inflammatory and other retinal cells in subretinal biopsies. All these factors also lead to a high level of subjectivity with respect to confidently reporting lymphoma just based on cytology. In this context, polymerase chain reaction (PCR)-based molecular methods might aid in the unbiased diagnosis of PIOL. Recently, *MYD88* L265P mutation has been suggested to be prevalent in most PIOL.[1]

Myeloid differentiation primary response 88 (*MYD88*) gene is located in chromosome 3p22.2 and it provides instructions for making the *MYD88* protein involved in signaling within immune cells. *MYD88* protein acts as an adaptor molecule involved in Toll-like receptors (TLRs) and interleukin-1 receptor (IL-1R) signaling pathway.[2,3] Following a stimulus from TLRs, activation of *MYD88* leads to increased downstream proinflammatory pathways such as NFκ-B activation and favors tumor-cell survival.

A single base change in the DNA sequence in the *MYD88* gene wherein adenine is substituted by guanine resulting in a specific amino acid mutation at position 265 (where lysine is substituted by proline) leads to constitutive activation of

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B-cells[4] and it is associated with various disease states. *MYD88* L265P somatic mutation is reported in over 90% of Waldenstrom macroglobulinemia,[5-8] 100% in lymphoplasmacytic lymphomas,[9] 14–30% in diffuse large B-cell lymphoma,[10] 33% in primary central nervous system (CNS) lymphoma,[11] about 3% in chronic lymphocytic leukemia,[12] 15% of splenic marginal zone lymphoma,[13] and immunoglobulin M (IgM) monoclonal gammopathy of undetermined significance[14] by whole genome, exome, or Sanger sequencing. The frequency of *MYD88* gene mutation in PIOL has been studied in a western cohort. A prevalence of 69% has been reported in B-cell vitreoretinal lymphoma (VRL) by Bonzheim *et al*.[1] and Raja *et al*.[15] has reported 82% positive in VRL and 86% positive in PIOL. In another independent study, two of three cases were positive for *MYD88* L265P mutation in PIOL.[16]

Diagnosis of PIOL cannot be made only by clinical features, investigations and microscopic evaluation. The manifestation of the disease can be either vitreal, as subretinal lesions, or both and may also involve the optic disc and retinal vessels. Vitreous opacities may be caused due to the reactive inflammatory cells in vitreous. Subretinal lesions may begin as small, yellow to white mounds, which may enlarge and expand and further coalesce to produce large yellow subretinal masses with brown pigmentation in the center known as "leopard skin pigmentation". The lesions may involve optic disc producing an optic nerve head swelling. Vasculitis with retinal hemorrhages can also be seen as a rare presentation. Sheathing of the vessels may be seen, which could be reactive or due to lymphoma cell infiltration.

Blurring of vision and/or floaters are presenting symptoms. Vitreous floaters long before PIOL is suspected and are usually due to normal degenerative changes or uveitis. The final clinical diagnosis of PIOL is based on the following observations: anterior segment showing anterior chamber cells as well as keratic precipitates.[17]

Anterior segment inflammation is usually absent or the anterior segment is usually quiet.[17] Lymphoma cells may grow along the Bruch's membrane under the retinal pigment epithelium. These may appear as creamy lesions with orange-yellow infiltrates deep to the retina.[18] Islands of pigment float on these deposits give rise to a characteristic 'leopard-skin' pigmentation. Subsequent primary CNS lymphoma occurs in 40–90% patients within a mean interval of 8–29 months.[18]

The difficulty lies in diagnosing the disease due to its uncommon occurrence and masquerading as uveitis. Patients may be initially treated with topical or systemic corticosteroids or both. Temporarily patients may get benefitted from steroids and thus delay the eventual diagnosis of PIOL. Because lymphomatous cells are responsive to steroids, the "uveitis" may improve, only to recur with a decrease in the dose of steroids or discontinuation of therapy. Although several studies have confirmed the prevalence and frequency of *MYD88* L265P mutation in PIOL, we believe a comprehensive comparison of the diagnostic utility of the *MYD88* L265P mutation analysis with cytology would be helpful to ascertain its role in routine laboratory diagnosis of PIOL.

Methods

Patient and samples

The study was approved by the Institutional Ethics Committee (IEC no. 670-2018P). Consecutive vitreous and subretinal biopsies from patients (n = 21) with intraocular inflammatory pathology with or without suspicion of PIOL, between May 2018 and May 2019 for histopathological analysis were included in the study. In total, 25 clinical samples were available for the analysis. This includes vitreous samples alone from 17 patients and paired vitreous and subretinal samples from four patients.

Cytology/Cell Block

Based on the availability of the samples, cytological smear was prepared on charged slides as either direct smear (when less sample volume) or cytospin (when enough sample volume) using SHANDON CYTOSPIN®4, Thermo Scientific. Smears were allowed to air-dry and fixed with 95% ethanol for 10 minutes. Then, the fixed smears were subjected to modified hematoxylin and eosin staining.[19]

Cell-blocks were prepared based on the cellularity of the samples. An equal volume of 95% alcohol and vitreous aspirate added directly into the tube and made it stand for 2–3 hours. Cells in the fluid formed a soft mass. Tubes were centrifuged to get the soft cell mass, which was removed by the applicator stick and 10% neutral buffered formalin (NBF) was added for fixation. The obtained cell mass was processed under routine tissue processing method and embedded in paraffin wax to make the cell block.[20]

The stained smears were mounted with DPX mounting medium and observed under a binocular microscope (NIKON ECLIPSE Ci-L). Images were captured with 20× and 40× objectives by using ScopeImage 9.0 software. Since the main pathologist had access to the patient's clinical details as well as other test details, to mitigate the bias, the microscopic images of the cytological samples were scored by two more pathologists who had equivalent ocular pathology expertise. All samples were reported by all three pathologists: one in-house and two external. The external pathologists were provided with the high-quality images of vitreous aspirate cytology and had been asked for their interpretation of the cytological analysis without any clinical information (blinded). The inter-rater agreement between the pathologists was ascertained. The results obtained were classified as concordant positive (cytological confirmation of lymphoma by all three pathologists); concordant negative (when all three pathologists cytologically ruled out lymphoma); and discordant (when one or more pathologies had a disagreement with the cytological results).

MYD88 L265P Mutation analysis

DNA extraction and MYD88 PCR

The DNA extraction was carried out in vitreous aspirate and subretinal biopsy (n = 25) using the QIAGEN DNA extraction kit. PCR was carried out to amplify the region flanking the *MYD88*L265P mutation. The primer sequences utilized were Forward primer 5′-GGG ATA TGC TGA ACT AAG TTG CCA C-3′ and reverse primer 5′-GAC GTG TCT GTG AAG TTG GCA TCT C-3′ which yielded a 726-bp amplicon.[5] PCR reaction was performed in a final reaction volume of 20 µl using EmeraldAmp GT PCR Master Mix (TaKaRa). The amplified products were visualized in 1% agarose gel using ethidium bromide.

Sanger sequencing

Cycle sequencing was done with the amplified PCR products using BigDye Terminator v3.1 cycle sequencing kit using the reverse PCR primer. The cycle sequenced products were

purified and sequenced using Applied Biosystems 3130 Genetic Analyzer.

Restriction fragment length polymorphism (RFLP)

To assess the *MYD88* L265P mutation status, we attempted to validate restriction fragment length polymorphism (RFLP) analysis as a quick alternate and cheaper method for PIOL diagnosis on all of the above samples. Briefly, PCR primers covering the mutation site were designed to amplify a 415-bp product. The forward and reverse primer sequences were 5′-AAT GTG TGC CAG GGG TAC TTA G-3′ and 5′-GAC GTG TCT GTG AAG TTG GCA TCT C-3′. The amplified PCR products were subjected to restriction enzyme digestion using BsiE1 (New England Biolabs, MA, USA) at 37°C for 4 hours. The mutated allele contains a BsiE1 site resulting in 278 bp and 137 bp fragments, whereas the wild-type allele showed a single band of 415bp.

Statistics

The diagnostic utility of the cytological and *MYD88* L265P mutation analysis was assessed in terms of the clinical parameters such as sensitivity, specificity, positive/negative predictive values and accuracy using final clinical diagnosis as the gold standard.[21,22] The numerical values of these parameters were calculated using standard formulae.

Results

The clinical characteristics of the patients are provided in Table 1. There were 12 (57.2%) males and 9 (42.8%) females. The average age was 57.2 years with a range of 23–88 years. Out of 21, 15 patients were clinically suspected to have PIOL [Table 2]. Upon follow-up, 9/15 15 patients were treated for PIOL and responded to treatment (henceforth confirmed as PIOL). The rest six patients were diagnosed for other infectious and inflammatory pathologies (such as *Mycobacterium tuberculosis* intermediate uveitis ($n = 3$), *Varicella Zoster virus* retinitis ($n = 1$), *Cytomegalovirus* retinitis ($n = 1$), and Scleritis ($n = 1$)); henceforth referred to as other intraocular pathologies (OIP).

Of the six patients who were not clinically suspected of having PIOL, one of the patients initially diagnosed with primary tuberculosis was confirmed to have PIOL after cytological and molecular diagnostic testing. The remaining five cases had either infectious or inflammatory etiology such as *Mycobacterium tuberculosis* intermediate uveitis ($n = 2$), varicella zoster virus retinitis ($n = 1$), endophthalmitis ($n = 1$), and sarcoidosis ($n = 1$).

Based on the final diagnosis, 12 patients with other OIP were confirmed as non-PIOL.

Cytological images were reviewed by three ocular pathologists individually and scored as mentioned in the methodology [Figure 1]. Overall concordance in the reporting, irrespective of the final clinical diagnosis between the pathologists were 14/21 (66.7%) cases and 15/25 (60%) samples. Six of the nine clinically confirmed PIOL cases were scored concordant positive; while the rest three carried discordance in the reporting. Of the 12 cases where seven were scored as concordant negative, four carried discordance in the reporting, and one case of *Mycobacterium tuberculosis* intermediate uveitis was scored as concordant positive.

The diagnostic utility of the cytological analysis was assessed using the final clinical diagnosis as the gold standard (clinical diagnosis is described in the introduction section in detail). The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were 66.7%, 58.3%, 54.5%, 70%, and 61.9%, respectively [Table 3].

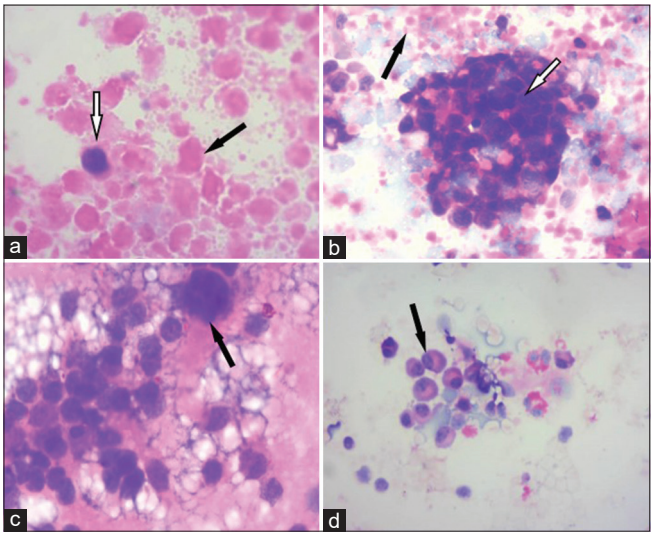

Figure 1: Different cytological patterns observed in PIOL post hematoxylin and eosin staining of intraocular cytological specimens. (a) Extensive necrotic cells (black arrow) with occasional lymphoma cells (white arrow). (b) Clumps of large atypical lymphoid cells (white arrow) with high nucleocytoplasmic ratio in a necrotic background (black arrow). (c) Atypical lymphoid cells (black arrow) with high nucleocytoplasmic ratio along with little or no necrotic cells. (d) Lymphoplasmocytic infiltrate with plasma cells (black arrow) along with little or no necrotic cells

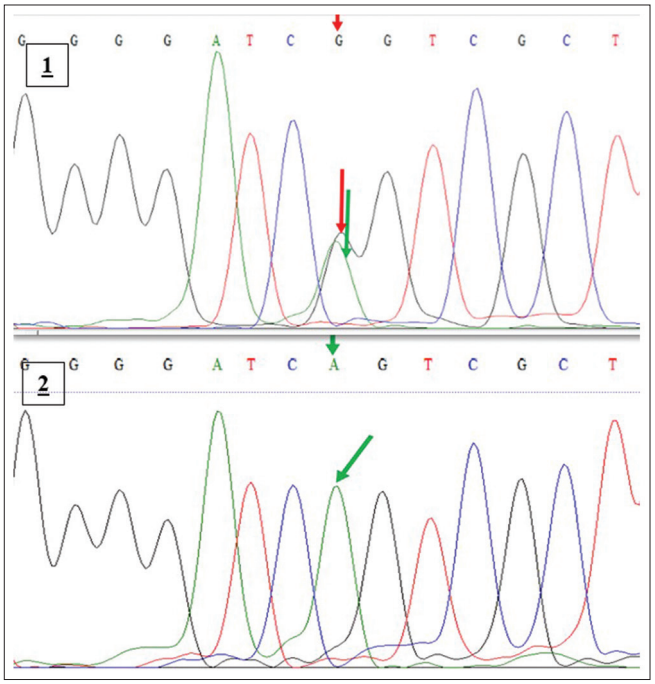

Figure 2: Detection of *MYD88* gene mutation status in PIOL patients by Sanger sequencing. 1) Heterozygous *MYD88* L265P mutation was observed in the vitreous sample of PIOL patient (VA12). Mutant allele (base G; red arrow) and wild-type allele (base A; green arrow) are represented and 2) Wild type *MYD88* sequence from the vitreous sample of cytomegalovirus retinitis patient (VA07)

Table 1: Clinical details of cytological analysis & *MYD88* L265P mutation status of 21 patients

Case No	Sample	Age/ Gender	Clinical Diagnosis		Inter Pathologists Cytological Analysis	*MYD88* L265P Mutation Status
			Actual Diagnosis	Final Diagnosis		
VA-03	Vitreous	57/F	PIOL	PIOL	Concordant (+ve)	Mutant
VA-04	Vitreous	63/M	PCNSL	PIOL	Concordant (+ve)	Mutant
VA-12	Vitreous	79/F	PIOL	PIOL	Concordant (+ve)	Mutant
VA-15	Vitreous	58/F	PCNSLO	PIOL	Concordant (+ve)	Mutant
VA-17	Vitreous	52/M	PIOL	PIOL	Concordant (+ve)	Mutant
VA-20	Vitreous	52/F	PIOL	PIOL	Concordant (+ve)	Mutant
VA-10	Vitreous*	69/M	PIOL	PIOL	Discordant	Mutant
VA-19	Vitreous	75/F	PCNSLO	PIOL	Discordant	Mutant
VA-09	Vitreous*	52/F	PIOL	PIOL	Discordant	Wild type
VA-05	Vitreous	23/M	MTB	OIP	Concordant (+ve)	Wild type
VA-02	Vitreous	64/F	VZV retinitis	OIP	Concordant (-ve)	Wild type
VA-07	Vitreous	27/M	CMV retinitis	OIP	Concordant (-ve)	Wild type
VA-16	Vitreous	67/M	Scleritis	OIP	Concordant (-ve)	Wild type
VA-08	Vitreous	68/F	MTB IU	OIP	Concordant (-ve)	Wild type
VA-01	Vitreous	54/M	MTB IU	OIP	Discordant	Wild type
VA-11	Vitreous*	71/F	Sarcoidosis	OIP	Concordant (-ve)	Wild type
VA-13	Vitreous	38/M	MTB Uveitis	OIP	Concordant (-ve)	Wild type
VA-14	Vitreous	67/M	VZV retinitis	OIP	Concordant (-ve)	Wild type
VA-06	Vitreous	89/M	Endophthalmitis	OIP	Discordant	Wild type
VA-18	Vitreous	40/M	MTB IU	OIP	Discordant	Wild type
VA-21	Vitreous*	34/M	MTB/PIOL	PIOL	Discordant	Mutant

PIOL- Primary Intraocular Lymphoma, PCNSL- Primary central nervous system lymphoma, MTB=Mycobacterium tuberculosis, IU- Intermediate Uveitis, VZV- Varicella Zoster Virus, CMV=Cytomegalovirus, OIP=Other Intraocular Pathologies. *Subretinal biopsies were also obtained from the patients for analysis

Table 2: Comparison of cytological analysis with final clinical diagnosis

Cytology	Final Clinical Diagnosis		Total
	True (PIOL)	False (No PIOL)	
Positive for PIOL*	06	05	11
Negative for PIOL#	03	07	10
TOTAL	09	12	21

*Refers to concordant positive results in cytology. #Refers either to concordant negative or discordant results in cytology. Final clinical diagnosis was considered as gold-standard for comparison

MYD88 L265P Mutation analysis

MYD88 L265P mutation analysis was carried out by both Sanger sequencing and PCR-RFLP on all the 25 clinical samples. Both Sanger sequencing [Table 1 and Figure 2] and PCR-RFLP [Figure 3] showed concordance in all 25 clinical samples. In addition, concordant results were observed with respect to paired vitreous and subretinal fluid obtained from four patients.

Of the nine clinically confirmed cases of lymphoma, all but eight cases carried a mutant allele. Of the 12 non-lymphoma cases, 11 revealed a wild-type allele. One of the cases previously confirmed clinically as *Mycobacterium tuberculosis* intermediate uveitis showed *MYD88* positivity, which on follow- up was confirmed as PIOL. Diagnostic utility of *MYD88* L265P mutation analysis revealed a sensitivity of 88.9%, specificity of 91.6%, positive and negative predictive value of 88.9% and 91.7%, respectively. Diagnostic accuracy of 90.5% was achieved with the mutation analysis, which shows the superiority of *MYD88* in both ruling in and ruling out PIOL.

Discussion

While clinical features help in suspecting the diagnosis and investigative tools help to differentiate it from other masquerades, it is the microscopic evaluation that forms the mainstay of diagnosis in such patients[17]. Approximately, 60–80% cases of PIOL are bilateral involvement and frequently mimics as chronic posterior uveitis.[18,23] While magnetic resonance imaging (MRI) can confirm the diagnosis of IOL with CNS involvement, to ascertain the clinical diagnosis of IOL without CNS involvement is highly challenging. The diagnosis of IOL becomes more difficult when the patient presents with intraocular mass or masquerade syndrome or have undergone treatment with steroids or immunosuppressant drugs. Although cytology is considered to be a gold standard with respect to the laboratory diagnosis of PIOL, the difficulty to diagnose PIOL predominantly by cytology is attributed to the following reasons.

a) Previous vitrectomy leading to lower cellular content of the vitreous resulting in non-convincing cytology results. **b)** Prior steroid treatment leading to the apoptosis of lymphoma cells. **c)** Predominance of inflammatory cells over lymphoma cells in case of vitreous aspirate. **d)** Sample processing methods (direct versus cytospin smear).

Besides the limitations with respect to the clinical specimen or other technical issues with respect to lack of

typical cells or possible variation in the cellular content of cell block sections, the interpretation of the cytology also relies on the expertise of the ocular pathologists reviewing the slides. To assess the inter-observer bias or subjectivity, we provided the cytology images of the intraocular samples to three ocular pathologists with equivalent expertise. The lower rate of concordance in the reporting suggested that cytological analysis of PIOL may be highly subjective and one cannot rely only on cytology results. In addition, the attributes of the diagnostic utility of cytological analysis in diagnosing PIOL were very low [Table 4] with an approximate accuracy of 59%.

Since PCR-based assays have been shown to be less subjective and more useful in establishing infectious etiology in intraocular fluids, we searched for PCR-based assays to improve the diagnosis of IOL. Since PCR-based sequencing for *MYD88* L265P was less complex than IgH rearrangement, we preferred to compare its diagnostic utility to aid the cytological analysis.

Our results suggested that *MYD88* L265P mutation analysis had a better diagnostic profile in terms of all the parameters such as sensitivity, specificity, and positive and negative predictive value as well as accuracy. We observed that the positivity rate of *MYD88* L265P in clinically confirmed cases of lymphoma was 80%, which is in par with those observed in previous studies[1,15], which ranges between 69% and 88%.

MYD88 L265P mutation was absent in one of the confirmed lymphoma cases (VA9), it is to be noted that cytological analysis also showed discordant results and was less informative. This case (VA9) had a classical clinical presentation of subretinal pigmented epithelial (RPE) deposits with leopard lesions, which allowed the clinician to decide on the therapy in lieu of negative results with both cytology and *MYD88* L265P mutation. In addition, immunohistochemistry of CD20 on subretinal biopsy turned out to be positive, confirming as PIOL

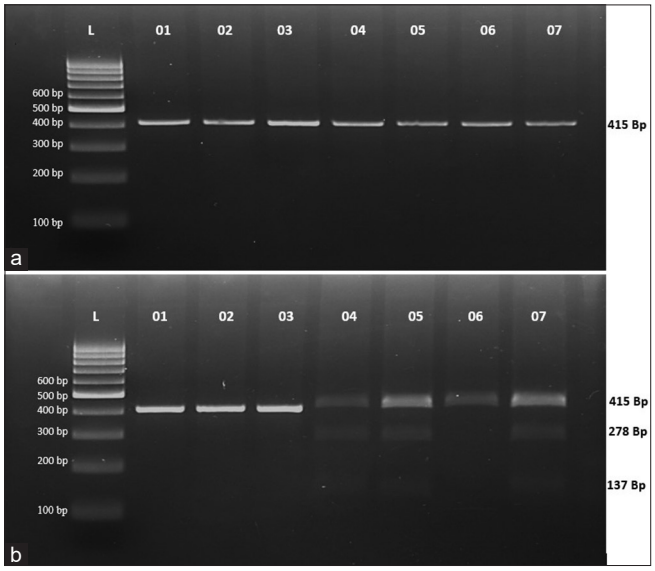

Figure 3: *MYD88* L265P mutation by PCR-RFLP using BsiE1 restriction enzyme. (a) PCR amplicon of 415 bp were observed in samples 1 to 7 on 1.2% agarose gel. (Lanes L-100 bp DNA ladder, 01-VA02, 02-VA07, 03-VA09, 04-VA03, 05-VA12, 06-VA14, 07-VA15). (b) PCR-RFLP analysis on 3.0% agarose gel showing either negative restriction digestion (single 415bp band) (Lanes 1-3, 6) or heterozygous *MYD88* L265P mutation [three bands of 415bp (wild type); 278 and 137 bp (mutant) (Lanes 4, 5, 7)]. Lanes L-100 bp DNA ladder, 01-VA02, 02-VA07, 03-VA09, 04-VA03, 05-VA12, 06-VA14, 07-VA15

In addition to these cases where *MYD88* failed to identify lymphoma, one of the cases (Case No. VA21); although classified as "false positive" in this manuscript, actually helped the clinicians to rule in lymphoma in a patient with primary tuberculosis. This patient had a history of tuberculosis and presented with subretinal granuloma in both eyes with subretinal gliosis at the macula. He was put on anti-tubercular treatment regimen category-2, however, with no improvement in the subretinal granuloma. PCR for *Mycobacterium tuberculosis* turned negative suggesting a different entity to be involved in the pathology and *MYD88* L265P mutation test by Sanger sequencing aided in ruling in lymphoma. Currently, the patient is being treated with rituximab and responding well to the treatment.

Owing to the higher cost, technical difficulties, and turn-around time involved with IgH gene rearrangement assay, *MYD88 L265P* gene mutation by Sanger sequencing provides to be a very useful alternative. In addition, to be useful in establishing a reliable diagnosis of lymphoma, we were able to reduce both the cost and turn-around-time to reporting the *MYD88* mutation status by adapting an already established PCR-based RFLP assay.[7,25] The PCR-RFLP showed 100% concordance with PCR-based sequencing results and could be adaptable in any basic molecular biological laboratory.

Since our hospital is a tertiary care center, all patients included in the study had been referred from other hospitals for further evaluation. All patients had been previously treated with steroids but were tapered off the dose before the vitreous biopsies were taken. Variation in sample preparation and steroid treatment did not affect the diagnosis of PIOL by Sanger sequencing. Hence, we suggest *MYD88 L265P* gene mutation test for the diagnosis of PIOL.

Table 3: Comparison of *MYD88* L265P mutation analysis with final clinical diagnosis

MYD88 L265P Mutation	Final Clinical Diagnosis		Total
	True (PIOL)	False (No PIOL)	
Mutant Allele	08	01	09
Wild-Type Allele	01	11	12
TOTAL	09	12	21

Final clinical diagnosis was considered as gold-standard for comparison.
PIOL: PIOL- Primary Intraocular Lymphoma

Table 4: Comparative diagnostic utility of Cytological analysis and *MYD88* L265P mutation analysis

	MYD88 L265 Status	Cytological Status
Sensitivity	88.9%	60.0%
Specificity	91.6%	58.3%
Positive predictive value	88.9%	54.5%
Negative predictive value	91.7%	70.0%
Accuracy	90.5%	61.9%

All the diagnostic parameters were calculated based on established formulae[23,24]

Conclusion

In conclusion, our study suggests *MYD88* L265P to be a highly reliable test for diagnosing PIOL in an ophthalmic tertiary care setting where most complicated, already treated, and non-responsive cases are referred. The major limitation is the smaller cohort on which the analysis has been carried out. A multicenter longitudinal study would aid in establishing the actual utility of *MYD88* L265P mutation analysis in PIOL.

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Conflicts of interest

There are no conflicts of interest.

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