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Isolation of Selected Epstein - Barr Virus Genes in Swab Samples of Suspected Cases

Lunjapikai Haokip[1*]; Kuppa Vagdevi[1]; Annette Angel[2]; Bennet Angel[2]; Vinod Joshi[2]; Monika Dheer[2]; Prigya Sharma[2]

Corresponding Author: Lunjapikai Haokip[*]

[1]Dept of Life Science, Sharda School of Bio-Sciences & Technology, Sharda University
[2] Centre of Excellence in Virology & Immunology, Sharda University, Greater Noida, UP, India.

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Abstract: The reported study has been undertaken to validate a molecular assay for isolation of key EBV proteins in clinical samples of saliva in suspected cases by employing laboratory techniques, namely Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis (AGE), etc. BamHI Z fragment leftward open reading frame 1 (BZLF1), BCLF1 (major capsid protein) or BC1, and Epstein-Barr nuclear antigen 1 (EBNA1) were selected as target genes due to their role in the viral replication cycle, latency, oncogenesis, and lytic reactivation.

BZLF1 is an immediate-early viral gene, also known as Zta, EB1, or ZEBRA. It is frequently used for the detection of EBV as it is a conserved gene. It plays an important role in converting the EBV from the latent to lytic stage and further leading to viral replication. This is the most studied gene. BCLF1 is a late gene involved in the formation of structural proteins, specifically the major capsid protein, in association with other genes like BFRF3, BDLF1, and BORF1. EBNA1 is a latent gene responsible for the virus's replication, maintenance in infected cells, and evasion from the immune system. All three genes are very important in EBV-related malignancies.

The molecular assay was carried out in 11 samples of suspected EBV cases, which showed the presence of at least one of the EBV proteins in all the samples. The BZLF1 gene was most prevalent, as 6 out of 11 samples were found positive for this gene, followed by the BCLF1 gene positive in 5 samples. EBNA1 protein was present only in 2 samples. The results showed good diagnostic utility of PCR to detect EBV genes.

Keywords: *EBV; Oral Carcinoma; EBV Genes; Malignancies; Molecular Assay; Viral Replication; PCR.*

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I. INTRODUCTION

Epstein - Barr virus (EBV) is the most prevalent virus present in almost every individual at some time in their lifetime, as a child or during adulthood. Globally, 95% of the individuals are infected with this virus. It is from the subfamily of gamma herpesvirus, an oncogenic virus which is associated with many diseases, related to the immune system, cancer, and autoimmune disorders, and may exist with many different viruses in one human system. It is prevalent from very childhood, regardless of age, sex, caste, etc. During childhood, it is asymptomatic, but with time, it causes Infectious Mononucleosis (IM) in the majority of cases [1].

The seroprevalence of EBV in India has a high prevalence rate, indicating past and present infection in many cases. Many studies show the presence of IgM at the start of infection, and further, the presence of IgG against latent proteins like EBNA after a gap of many years, indicating the existence of a latent cycle in the cells. In the serological tests, the VCA is expressed during EBV infection, which can be targeted by antibodies, showing the past and present form of infection. Every human, except for very few individuals, is infected at an early stage in life with EBV. EBV remains either in a latent or lytic form in an individual.

To detect the presence or absence of EBV genes in humans, there are several genes like LMP1, EBNA1, EBNA2, BZLF1, BRLF1, Viral Capsid Antigens (VCA), EBER1, and EBER2 are used in the diagnosis of EBV from

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various clinical samples. All the above genes are critical in the life cycle of EBV. For the persistence of EBV, it is the EBNA1 (Epstein-Barr Nuclear Antigen 1) and EBNA2 (Epstein-Barr Nuclear Antigen 2) that play a crucial role; in addition, EBNA2 transforms B cells. BZLF1 and BRLF1 (Latent Membrane Protein 1), known as an immediate-early gene,are responsible for the reactivation of the lytic cycle. EBER1 and EBER2 (Epstein-Barr RNA-encoded) are small non-coding RNAs found in abundance in the infected cells [2].

Techniques like PCR (Polymerase Chain Reaction), sequencing are used for the detection, subtyping, and genetic diversity of EBV types. Among all these, PCR is one of the common methods for the detection of various genes of EBV. Immunohistochemistry (IHC) can be used to identify EBV antigens in tissue samples against the circulating antibodies to detect and visualize specific antigens [3].

Table 1 EBV Genes and their associated cancer

Cancers caused	EBNA1	BZLF1	BCLF1
Nasopharyngeal Carcinoma (NPC)	Promotes cell survival and immune evasion.	Promotes viral reactivation in Neural Progenitor cells.	Increases immune suppression by interfering with antigen presentation.
Hodgkin's Lymphoma (HL)	EBNA1 helps maintain the viral genome in Reed-Sternberg cells [4].	Expression can trigger lytic cycles in some HL cases.	Contribute to chronic inflammation and tumor microenvironment remodeling.
Burkitt's Lymphoma (BL)	Supports persistence of EBV infection.	Sporadic BZLF1 reactivation can promote genomic instability[5].	BCLF1 expression in lytic phases may contribute to immune evasion and disease progression.
Post-Transplant Lymphoproliferative Disorder (PTLD)	EBNA1-driven EBV latency leads to uncontrolled B-cell proliferation.	BZLF1 drives viral replication, leading to uncontrolled B-cell proliferation.	BCLF1 can promote uncontrolled B-cell proliferation[6].
Gastric carcinoma (EBV-associated gastric cancer, EBVaGC.	Approximately 10% of gastric cancers globally are EBV+ve and express EBNA1.	BZLF1 is detected in early and late-stage EBVaGC, indicating intermittent viral reactivation.	-

II. MATERIAL METHODS

Eleven suspected oral patient's swabs were taken for EBVgene detection from Sharda Hospital, Greater Noida. The stepwise details for the collection and further assays are as follows:

A. Patient's Recordand Sample Collection:

The symptoms of the patients were recorded along with other signs conveyed by them at the time of admission to the hospital. The patients with different oral problems were informed about the consent form, and then only the swab samples were taken, and the Information sheet was signed by them. Patient's personal and clinical details such as name, age, gender, address, cause of admission to the hospital or visiting the doctor, duration of illness, signs, etc.,were recorded with their consent. The swab samples were carefully collected and stored in 15ml tubes containing VTM (viral transport medium) following the standard procedures and bio-safety measures to ensure accuracy and reliability of the assay to be performed further. The samples were aliquoted and stored at 80°C.

*B. DNA Extraction***:**

The viral nucleic acid was extracted from the swab samples using the QIMP viral DNA mini kit. 200 µl of the samples were taken, vortexed, and then centrifuged for 10 minutes at 3000 rpm for 4°C. Then, 200 µlof lysis buffer and 20 µl of proteinase K were added to the same tube containing the samples and then vortexed for 15 seconds. Samples were incubated at 60°C for 10 minutes. Then 200 µl of 100% ethanol was added to the tube containing samplesand centrifuged at 8000 rpm for 1 minute at room temperature. All the solution was transferred to the spin column tube, and 500 µl wash buffer 1 was added, and then centrifuged at 8000 rpm for 1 minute at room temperature. The eluted liquid was discarded. Then,wash buffer 2 was added, followed by centrifugation at 14000 rpm for 3 minutes. Again, the eluted liquid was discarded. A final dry spin was performed at 14000 rpm for 1 minute at RT. Now the spin column was transferred to a 1.5 ml tube, and 60 µl elution buffer was added to the spin column. It was incubated for 2 minutes at RT and centrifuged at 8000rpm for 1 minute. Samples were labeled properly and stored at -80°C.

C. Polymerase Chain Reaction (PCR):

EBV viral presence was detected using conventional Polymerase chain reaction (PCR) with the Himedia thermocycler system. Primers specific to major viral capsid protein (BCLF1), latent protein (EBNA1), and immediate early protein (BZLF1) were used. Each reaction mix included buffer of 6.25µl, enzyme 0.5µl, MgSO4 0.25µl, Forward primer 0.5µl, Reverse primer 0.5µl. Reaction mixes were prepared in a BSL Class II hood. 5µl of the extracted DNA was added to each well. The temperature settings in the Thermal cycler were - an initial denaturation of 1 cycle at 94°C for 3 minutes, followed by 50 cycles of denaturation at 94 °C for 15 seconds, annealing and extension at 60°C for 30 seconds, and a final cooling at 4 °C for 5minutes.

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D. Agarose Gel Electrophoresis:

Agarose Gel Electrophoresis was performed to check whether our viral genome had multiplied using specific primers. AGE is a standard lab procedure to separate DNA according to their size. Concentration of both 1% and 2% Invitrogen agarose was prepared in Tris-acetate-EDTA Buffer by adding Ethidium bromide (EtBr) to a final mixture that makes DNA visualization in (Ultraviolet) UV light. Samples were added to the wells. Running TAE buffer was filled in the tank, and gel was put in the tank. The gel was run at 100 volts for 30 minutes. The result was observed under a UV transilluminatoror a Gel documentation system.

III. RESULTS

From the 11 suspected oral disease patients, we could see, all were positive for EBV with different genes.After running the Agarose Gel electrophoresis for each swab sample for 3 sets of genes, i.e., BCLF1, BZLF1, and EBNA1; the following results were observed for the various genes.

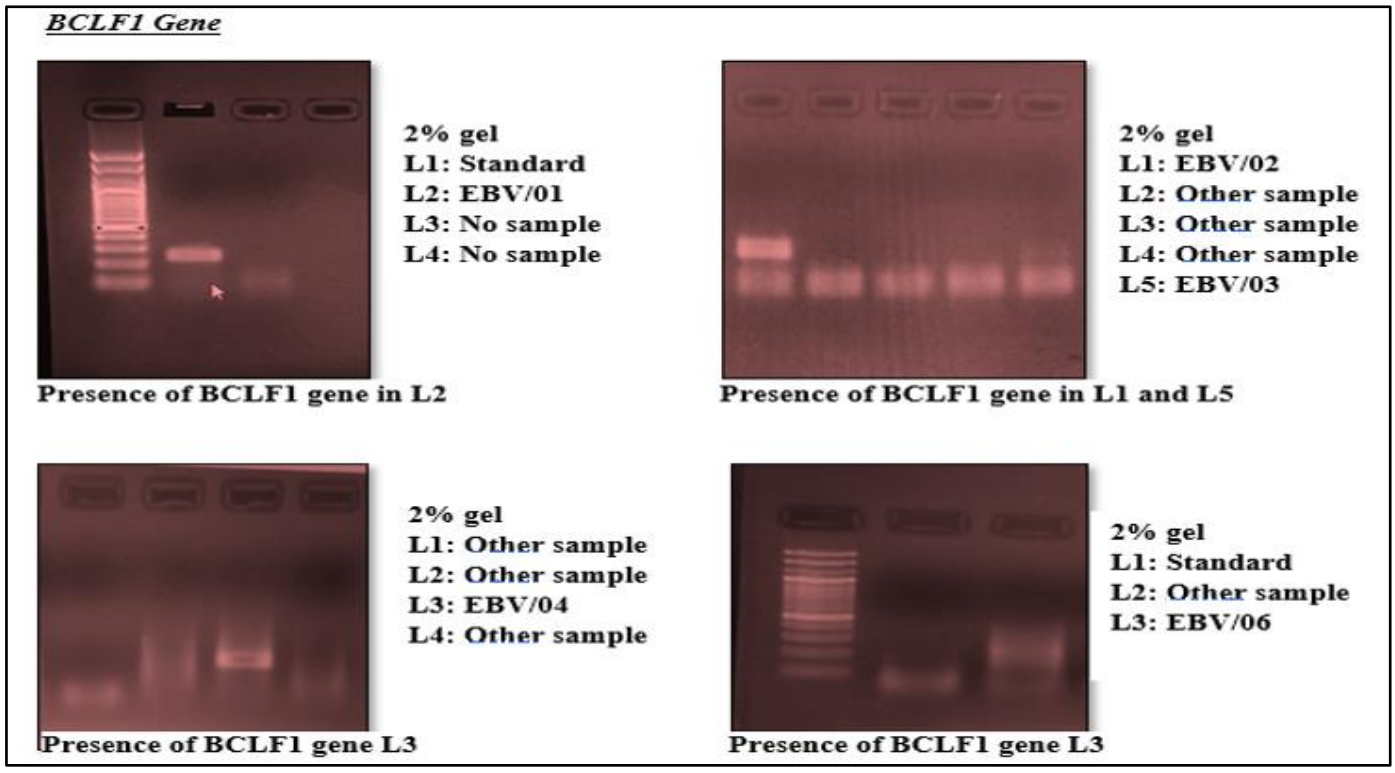

Fig 1 Presence of BCLF1 Gene in Agarose Gel Electrophoresis

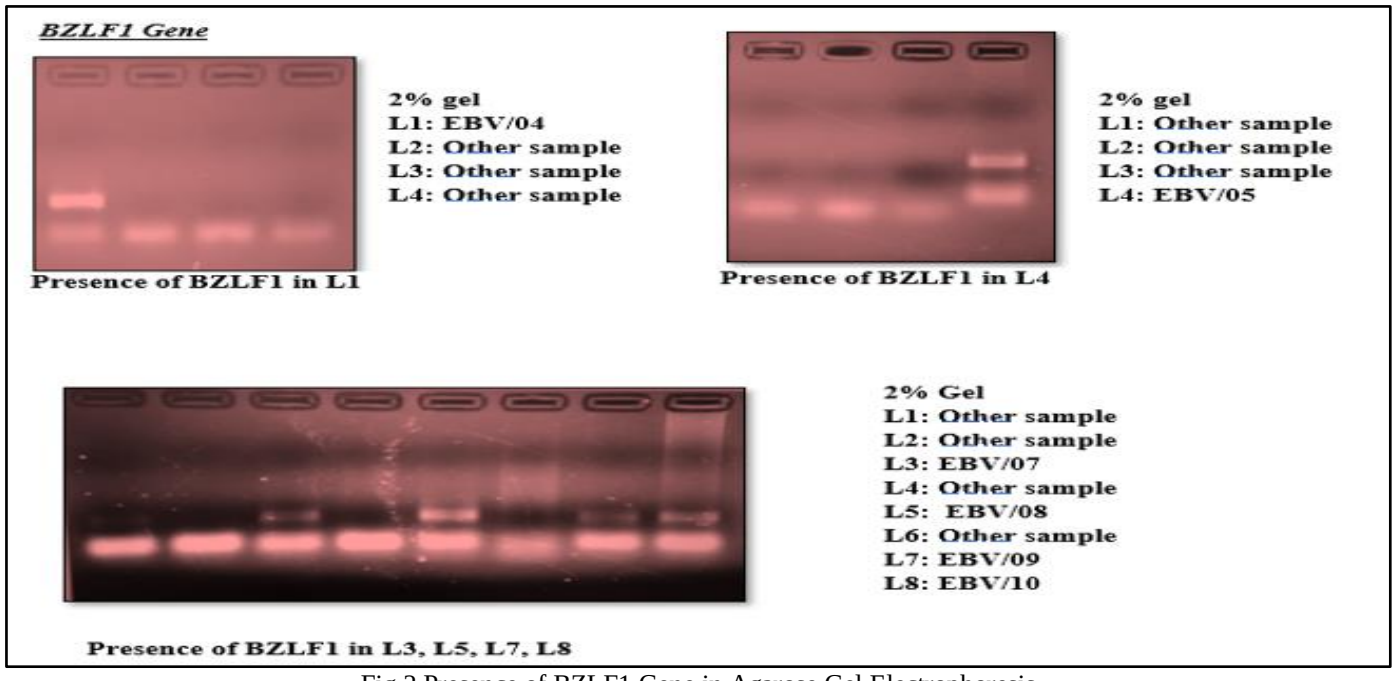

Fig 2 Presence of BZLF1 Gene in Agarose Gel Electrophoresis

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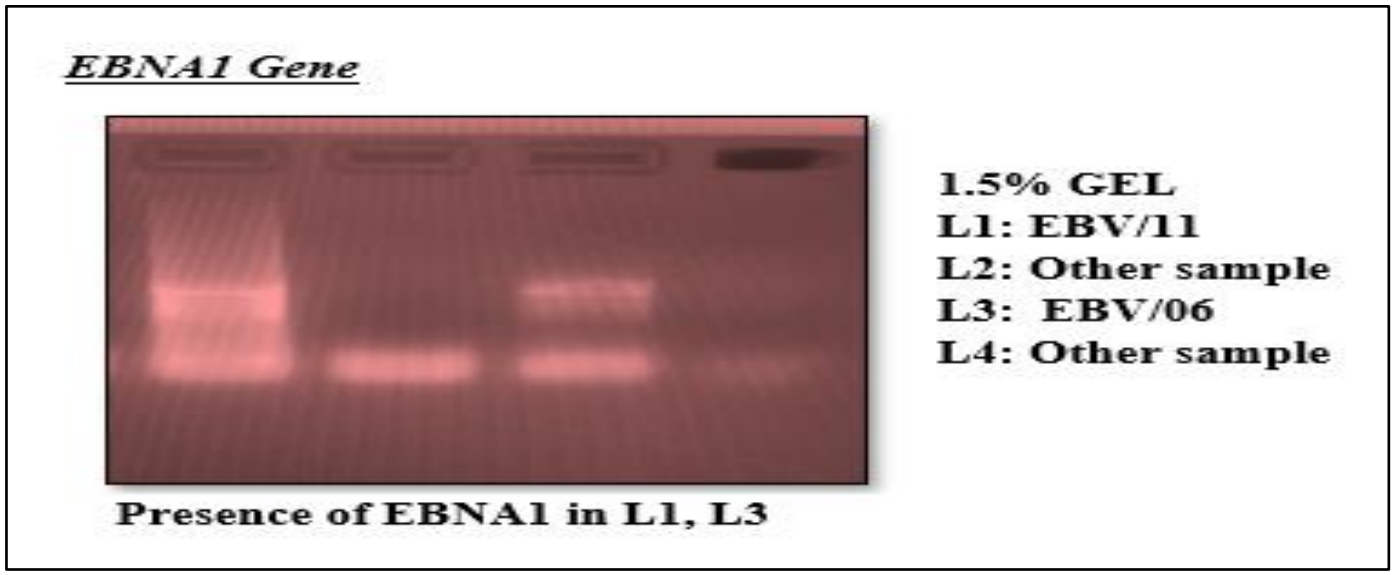

Fig 3 Presence of EBNA1 Gene in Agarose Gel Electrophoresis

Table 2 Summary of 3 Genes with Diseases Predicted for the Patients

S.No.	Sample Code	Name of the Diseases	BCLF1 Gene	BZLF1 Gene	EBNA1 Gene
1	EBV/1	Squamous cell carcinoma	Yes	-	-
2	EBV/2	Smoker's palate	Yes	-	-
3	EBV/3	Homogenous leukoplakia	Yes	-	-
4	EBV/4	Smoker's palate	Yes	Yes	-
5	EBV/5	Squamous cell carcinoma	-	Yes	-
6	EBV/6	Oral Submucous Fibrosi (OSMF)	Yes	-	Yes
7	EBV/7	Smoker's melanosis	-	Yes	-
8	EBV/8	Leukoplakia, smoker's palate	-	Yes	-
9	EBV/9	Leukoplakia	-	Yes	-
10	EBV/10	Oral Submucous Fibrosi (OSMF)	-	Yes	-
11	EBV/11	Squamous cell carcinoma	-	-	Yes

The above data in the Table 2 shows the disease as confirmed clinically associated with different oral disorders including cancer and benign cases. From the eleven patients 3 were having Squamous Cell Carcinoma, 3 were detected with Smoker's Palate, 3 were suffering from Leukoplakia, 2 were detected with Oral Submucous Fibrosis (OSMF) and 1 was detected with Smoker's melanosis.When analyzing the genes of EBV, we could see, 6 were positive with BZLF1 gene, 5 were positive with BCLF1 and 2 were positive for EBNA1. One patient showed the presence of two genes together i.e., BCLF1 and EBNA1 suffering from Oral Submucous Fibrosis (OSMF). One patient showed 2 diseases at the same time i.e., Smoker's Palate and Leukoplakia had BZLF1 gene of EBV indicating latency stage in the patient.The data clearly indicates the presence of EBV with cancer in three cases suffering from Squamous cell carcinoma (SCC).

Table 3 Personal and Clinical Details of the Patients with Oral Disorders

S.No.	Sample Code	History	Symptoms	Diseases
1	EBV/1	Tobacco chewing	Difficulty in opening mouth, swelling in left region, pain, teeth sensation	Squamous cell carcinoma
2	EBV/2	Usage of Bidi, Alcohol occasionally	Lesion on left side of cheek (Greyish pigmentation in mouth)	Smoker's palate
3	EBV/3	Smoking habit, Alcohol occasionally	Dirty teeth, Greyish and whitish pigmentation on below teeth	Homogenous leukoplakia
4	EBV/4	Usage of Bidi	Teeth stained, pigmentation on palate and buccal	Smoker's palate
5	EBV/5	Usage of Bidi, Alcohol occasionally	Left tongue growth, teeth stained. Ulcer proliferative growth below left border of tongue 2x1 cm ,whitish surface ,irregular firm does not bleed on touch	Squamous cell carcinoma
6	EBV/6	Usage of Gutka	Difficulty in opening mouth, dirty teeth	Oral Submucous Fibrosis

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7	EBV/7	Tobacco, Bidi, and ghutka, Alcohol occasionally.	Dirty teeth, Black patches on both side of cheeks	Smoker's melanosis
8	EBV/8	Usage of Bidi , Alcohol occasionally	Patient had no teeth. Patient came for replacement	Leukoplakia, Smoker's palate
9	EBV/9	Chronic Bidi smoker, artificial teeth attached	White patches present below the tongue, teeth started falling.	Leukoplakia
10	EBV/10	Usage of Bidi, Alcohol occasionally	Injury on mouth right side, pus secretion and maggots. Very deep depression. Difficulty in opening mouth, Dirty teeth.	Oral Submucous Fibrosis
11	EBV/11	Usage of Bidi, Alcohol occasionally	Lump on left side of lip. Itchy allergy	Squamous cell carcinoma

Table 3 shows three patients having association of EBV and Squamous cell carcinoma. Two patients show signs of Oral Submucous Fibrosis (OSMF) which is considered a precancerous stage. OSMF is linked to chewing of tobacco and italso leads to difficulty in opening the mouth which may further lead to squamous cell carcinoma. Other diseases like Oral Submucous Fibrosis (OSF), Leukoplakia and Smoker's palate may lead to Squamous cell carcinoma in near future looking on to the history of each patient, wherein they are continuously chewing tobacco, taking alcohol and bidi, etc which may assist different viruses to attack the human system especially EBV and in turn weakening immune system.

IV. CONCLUSION

We report the results of selected gene isolation in salivary samples of clinically suspected EBV patients for developing robust diagnostic molecular markers for early detection of viral genes. Present paper reports details of PCR isolated genes, namely BCLF1, EBNA1 and BZLF1. As reported by number of studies that EBV infects allindividuals nearly at any stage of their life,our data also are in conformity of earlier studies which showed presence of EBV genes in all the clinically suspected cases. As EBV has been seen to be present and associated with many other disorders, isolation of infected genesby PCR could be one of the accurate methods to detect the confirmatory presence of EBV in suspected samples.

As EBV has two cycles i.e., latent and lytic, therefore even though if both the genes of lytic or latent are expressed in our samples, it does not indicate that this gene is present only. The presence of the genes is the indication of the gene present right at the time of collection of the swab and replication cycle going during time swabsample containing the epithelial cells or B-cells was taken.

Here in our samples, we could see the presence of one of the genes from the three genes attemptedin the suspected samples, indicating the presence of EBV in all samples. The BZLF1 gene was mostly found in majority of samples, followed by BZLF1 and lastly EBNA1. BZLF1 the immediate early genewas identified in all the diseases whether cancerous, precancerous or benign, as in the case of Squamous cell carcinoma (SCC), Oral Submucous Fibrosis (OSMF) and Smoker's melanosis. This could help us to understand that in future the benign conditions may lead to cancerous stagesassociated with EBV.

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