بسم الله الرحمن الرحيم

Shendi University
College of Graduate Studies &Scientific Research

Molecular and Immunohistochemical Detection of Epstein - Barr Virus among Sudanese patients with Lymphomas and Nasopharyngeal Carcinoma in Khartoum State –Sudan

A thesis Submitted for ***PhD Degree*** *in Histopathology & Cytology*

By
Ibrahim Bakhit Yousif Elemam
B. Sc Honours degree(Histopathology and Cytology)
(University of Shendi 2003)
M.SC in (Histopathology & Cytology)
Sudan- University of Sciences and Technology-2009

Supervisor
Dr: Yousif Mohammed Yousif
MBBS. Faculty of Medicine (University of Khartoum
MD in Otorhino Laryngology. Faculty of Medicine (University of Khartoum
Associated professor-faculty of Medicine –Shendi University

Co-supervisor
Dr: Mohammed Sideeg Abd Elaziz
B.Sc,M.Sc, PhD. In Histopathology &Cytology-(SUST)
Associated professor –Sudan University for Sciences and Technology

(2015)

بسم الله الرحمن الرحيم

الآية

قال الله تعالى:

﴿ يَا أَيُّهَا النَّاسُ قَدْ جَاءتْكُم مَّوْعِظَةٌ مِّن رَّبِّكُمْ وَشِفَاء لِّمَا فِي الصُّدُورِ وَهُدًى وَرَحْمَةٌ لِّلْمُؤْمِنِينَ... ﴾

صدق الله العظيم

الآيـة 57 فـي سورة يـونـس

Dedication

-To my mother,

who taught me meaning of life.

-To my dear father,

who gave me love and respect.

-To my brother and sisters,

who bring happiness to my life.

-To my teachers,

who led me to the way of success.

-To my friends and colleagues,

I dedicate this study.

Acknowledgements

First of all I thank the Almighty Allah who helped me to complete this study. I would like to express endless thanks to my supervisor, Prof, Yousif Mohammed Yousif for his great efforts and guidance. I would like also to thank my co- supervisor Dr. Mohammed Sideeg for his great helps and endless support, great thank to Prof, Rashid Al Tayeb for his great helping.

I thank the staff and my college of the Faculty of Medical Laboratory Sciences.

I would like to Thank the work team of Radiation Isotope Center Khartoum (RICK) especially Dr. Nada Saleh, also my deep thanks extended to staff of ALzaharawi lab specially Dr Mogtaba.

I would like to thank Dr. Mohammed El Hassan Tayeb, who helped me in the statistical analysis for this search. I would also like to thank Dr. Mohammed Altegani.for this greater helping, my thanks extend also to the histopathologist, Dr. Hatim Mahmoud Ahmed who helps me in the confirming the diagnose of cases.

Also I would like to thank my friend, Dr.Moataz Ibrahim, Dr.Mohammed Abdalgadir for their greater effort and support.

Abstract

Epstein Barr virus is a very common virus that is already infecting high percent of population worldwide and persisting for the lifetime in the host. It is usually acquired in early childhood in developing countries and results of several studies have suggested a probable etiologic association between Epstein-Barr virus (EBV) and lymphoma and nasopharyngeal carcinoma; multiple EBV proteins can be expressed in infected lymphocytes, among which Latent membrane protein-1(LMP-1) is thought to be most important for transformation, therefore, the aim of this study was to investigate the association of LMP-1 EBV in theses tumors in Sudan

This retrospective and prespective study was conducted in Khartoum State – Sudan , the geographical and histoclinical data were achieved from the patients files , and Formalin –fixed paraffin embedded biopsies of one hundred and thirty five (135) of malignant lymphoma and nasopharyngeal carcinoma tissues (case group) and normal and reactive tissues (control group) , samples were used to investigate the positive rate of EBV, by anti (LMP-1) Immunostain according to Ventana auto stainer machine protocol (Ultra View™) and Standard Strepto Avididin Biotin (Thermo Fisher) protocol , and by polymerase chain reaction (PCR) , The PCR primers targets the EBV gene encoding the viral latent membrane protein one (LMP-1) ware applied in the study. The data was analyzed, using the statistical programs software Statistical Package for the Social Sciences (SPSS) version (11.5), Chi square test and different statistical measures were calculated.

Positive (LMP-1) Immunostain reaction for EBV, were reported in (32%) (34/105) among malignant tissues, compared with (13%) (4/30) of nonmalignant tissue, the (P.value= 0.04) ; while the positive PCR for EBV were reported

(43.8%) (46/105) in malignant tissues, compared with (23.3%) (7/30) in non malignant tissue, the (P.value= .0.043).

Regarding lymphoma, LMP-1 immunohistochemistry, positive rate was (45%) (5/11) for HL, and (31%) (18/59) for non Hodgkin lymphoma, there was no statistically significant difference between HL and non HL existed as regard to association of EBV as the (P.value= 0.333). The same finding but slightly higher was obtained by PCR (63.6) (7/11) and (37.3%) (22/59), for HL and non HL respectively. Also the study showed that, the malignant lymphomas had higher incidence in male than female and most commonly affected age group was children and young adults and the most predominant subtypes was Non Hodgkin lymphoma.

Considering nasopharyngeal carcinoma, males were more affected than females, the most commonly affected age group was middle age and elderly and the predominance subtype was non keratinized squamous cell carcinomas.

As regard to histological subtypes of NPC, the PCR detected EBV DNA in (100%) (9/9), (43%) (3/7) and (24%) (5/19) of undifferentiated squamous cell carcinoma , keratinized squamous cell carcinoma and in Non keratinized squamous cell carcinoma respectively ,(P.value =0.001) , while the LMP-1 immunostain reveal positive rate of EBV of (55%, 28% , 21%) for the above subtypes , with (P.value= 0.182,)

The study concluded that, there is sufficient evidence for the carcinogenicity of EBV in the causation of lymphomas and nasopharyngeal carcinomas, also the study concluded that PCR was more sensitive in detecting EBV. The study recommended to adopt EBV screening program, and to conduct further studies to find out whether the carcinogenicity of EBV need co-infection or not.

ملخص البحث

يعتبر فيروس الEBV من أكثر الفيروسات انتشارا على نطاق العالم, حيث يصيب الاطفال فى الغالب الاعم خصوصا فى الدول النامية , ترجح الكثير من نتائج الدراسات البحثية وجود علاقة سببية بين وجود فيروس (EBV) وسرطان الغدد اللمفاوية و سرطان البلعوم الانفى. لهذا السبب اجريت هذه الدراسة فى السودان للتحرى عن اثبات او نفى هذه العلاقة .

اجريت هذه الدراسة الاسترجاعية الآنية فى السودان(ولاية الخرطوم) , حيث جمعت المعلومات الجغرافية والاكلينيكية من سجلات وملفات المرضى, ومن ثم اخذت الخزعات النسيجية المحفوظة فى البرافين من الارشيف المختبرى, حيث تم اخذ مائة وخمس وثلاثون خزعة وكانت على النحو التالى: سبعون من سرطان الغدد اللمفاوية وعشرون من الغدد اللمفاوية الطبيعية والنشطة كما اخذت خمس وثلاثون خذعة نسيجية من سرطان البلعوم الانفى وعشرة من النسيج طبعى للبلعوم الأنفى, للكشف عن وجود هذا الفيروس ,اجريت تقنيتان نسيجيتان وهما تقنية المناعة الكيميائية النسيجية حسب مفهوم شركة ثيرمو وتقنية البيلوجيا الجزيئية لمضاعفة الحمض النووى حسب مفهوم شركة انترون, تم تحليل النتائج بطريقة كاى التربيعى بواسطة البرنامج الاحصائى للحزم الاجتماعية.

كشفت تقنية المناعة النسيجية عن وجود الفيروس بنسبة(32%) (105\34) من سرطانات الغدد اللمفاوية وسرطان البلعوم الانفى , فى حين كانت نسبة تواجده فى الانسجة غير السرطانية (عينة السيطرة) بنسبة (13%) (30\4) والقيمة الاحتمالية للارتباط كانت (O.043) , بينما النتائج البيلوجية الجزيئية لمضاعفة الحمض النووى للفيروس كانت على النحو التالى(43.8 %)(105\46) لعينة الدراسة و (23.3%)(30\7) لعينة السيطرة والقيمة الاحتمالية للارتباط كانت (0.04) مما يدل على وجود فروقات معنوية ذات دلالة إحصائية.

مايلى سرطان الغدد اللمفاوية: كانت نسبة تواجد الفيروس فى هذا السرطان هى (45%) (5 \11) لسرطان (HL)ونسبة (31%) (18\59) لسرطان (NHL) حسب تقنية المناعة الكيميائية النسيجية و اما طريقة كشف الحمض النووى لنفس السرطانات سابقة الذكر كانت (63.6% و 37.3%) ل(HL) و (NHL) على التوالى , حيث لاتوجد فروقات معنوية ذات دلالة احصائية إذ ان القيمة الاحتمالية للارتباط كانت (0.333), اوضحت الدراسة ايضا أن سرطان الغدد اللمفاوية اكثر تواجدا فى الرجال ودائما يصيب الاطفال ومتوسطى الاعمار, كما أن ال (NHL) هو النوع الاكثر إنتشارا.

مايلى سرطان البلعوم الانفى, فإن هذا السرطان أكثر إنتشار بين الرجال , ودائما ما يصيب متوسطى الاعمار وكبار السن, اوضحت الدراسة كذلك ان سرطان الخلايا الحرشفية غير المتقرنة هو أكثر الانواع النسيجية انتشار.

عند مقارنة ترافق فيروس الEBV مع الانواع النسيجية لسرطان البلعوم الانفى , اظهرت تقنية مضاعفة الحمض النووى, فروق ذات دلالة احصائية للاارتباط (0.000) الفيروس بدرجات متفاوتة مع تصنيفات السرطان النسيجية , حيث كان تواجده بنسبة (100% و 43% و 24%) لسرطان الخلايا الحرشفية غير المتمايز وسرطان الخلايا الحرشفية غيرالمتقرنة وسرطان الخلايا الحرشفية المتقرنة, على التوالى, بينما كانت نسبة تكرار الفيروس بإسعمال تقنية المناعة الكيميائية النسيجية لنفس الانواع آنفة الذكر كالآتى (55%و28% و21%) مع عدم وجود فروقات ذات دلالة احصائية حيث ان القيمة الإحتمالية كانت (0.182).

هنالك دلائل كافية تشير لوجود علاقة بين الاصابة بفيروس الEBV وسرطان الغدد اللمفاوية والبلعوم الانفى و اكدت الدراسة على ان تقنية تضاعف الحمض النووى للفيروس كانت اكثر حساسية من الطريقة المناعية الكيميائية النسيجية للكشف عن ذات الفيروس, أوصت الدراسة بالقيام بى برنامج مسح عام للكشف عن الفيروس وسط فئات المجتمع, اوصت الدراسة بإجراء دراسات مستفيضة لتوضح عما إذا كان هذا الفيروس يحتاج لاخماج مساعدة للقيام بعملية التسرطن ام لا.

List of abbreviations

Abbreviations	Meaning
AID	Activation Induced Deaminase
ALDs	Associated lymphoproliferative disorders
APCs	Antigens presenting cells
ATM	Axiatelangiectasia mutated
BL	Burkitt's lymphoma
cHL	Classical Hodgkin's lymphoma
CNS	Central nervous system
T cell	Cytotoxic T lymphocytes
DAB	3,3'-Diaminobenzidine
DAPK	Death associated protein kinase
DC	Dendritic cell
DCUs	Deep cortical units
DLBCL	Diffuse large B-cell lymphoma
DPX	Distyrene Plasticizer Xylene
EAgs	Early Antigens
EBER	Epstein Barr Encoded RNA
EBNA	Epstein-Barr Nuclear Antigen
EBV	Epstein-Barr virus
ELISA	Enzyme linked Immuno Sobbent Assay
FDCs	follicular dendritic cells
FFEP	Formalin fixed embbed paraffin
FRCs	Fibroblastic reticular cells
H&E	Hematoxylin and Eosin
Hcl	Hydrochloric acid

HCV	Hepatitis C virus
HHV-4	Human herpes virus 4
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HLA	Human Leukocyte Antigen
HSCT	Hematopoietic stem cell transplantation
HTLV-1	Human T-cell leukemia virus-1 type
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISH	In situ hybridization
LCLs	Lymphoblastoid cell lines
LMP	Latent membrane protein
LPHL	Lymphocyte predominant Hodgkin's lymphoma
MHC	Major Histo Compatibility
NASBA	Nucleic acid sequence-based amplification
NASBA	Nucleic acid sequence-based amplification
NCI	National cancer institute
NDMA	N-nitro di methyl amine
NHLs	Non-Hodgkin's lymphomas
NPC	Nasopharyngeal Carcinoma
NPIP	Nnitrospiperidine
NPYR	N nitrospyrrolidene
NPYR	N nitrospyrrolidene
PBS	Phosphate buffer saline
PTLDs	Post-Transplant Lymphoproliferative Disorders

RICK	Radio Isotope Centre Khartoum
ROS	Reactive Oxygen Species
R-S	Reed-Sternberg
SCC	Squamous cell carcinoma
SCID	Severe combined immune deficiency
SCR	Sudan cancer registry
SPSS	Statistical package for social and sciences
TBS	Tris buffer saline
TSGs	Tumor suppressor genes
VCa	Viral capsid
WHO	World Health Organization

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1.1. Introduction

1.1.1. Lymphoma:

Lymphoma is a cancer arising in the lymphatic system, lymphoma, is the most commonly occurring blood cancer. Approximately 1,000 people worldwide are diagnosed with lymphoma every day. The cells affected by this disease are part of the body's immune system. In 2013, the American Cancer Society estimates that 79,030 new lymphoma cases were diagnosed and 20,200 cancer deaths due to lymphoma was occur in the United States (Cancer Facts and Figures, 2010)

1.1.2. Lymphoma: Types

1. Hodgkin's Lymphoma (Hodgkin's disease)
2. Non-Hodgkin Lymphoma
3. Burkitt's Lymphoma

1.1.2.1. Hodgkin's Lymphoma

Cancer cells of these patients are usually had an abnormal B-cells referred to as Reed-Sternberg (*R-S*) cells. Although less commonly observed, R-S cells may also develop from T-cells (Long & Versea, 2006) R-S cells most often develop in lymph nodes located in the upper body regions and spread to neighbouring lymph nodes via lymphatic vessels. There are two distinct types of Hodgkin's lymphoma: classical and non-classical Hodgkin's lymphoma .Classical Hodgkin's Lymphoma: This lymphoma has features of R-S cells with a classical appearance. It may be diagnosed as Nodular Sclerosis Hodgkin Disease, Mixed Cellularity Hodgkin's Disease, Lymphocyte-Rich Hodgkin Disease or Lymphocyte-Depleted Hodgkin Disease. Non-classical Hodgkin's Lymphoma: This lymphoma features larger cancer cells that are variants of R-S cells and is most often found in the nodes of the upper body, arms and neck (Learn About Lymphoma. 2010).

1.1.2.2. Non-Hodgkin Lymphoma:

The cancerous cells of non-Hodgkin lymphoma patients may be either T or B cells. In the United States, approximately 15% of cases of non-Hodgkin lymphoma develop from T lymphocytes and 85% develop from B lymphocytes. (.Normal white blood cells may develop into over thirty different variations of abnormal cells, each classified as a distinct type of non-Hodgkin lymphoma. (Learn about lymphoma. 2010)).

1.1.2.3. Burkitt's lymphoma:

Burkitt's lymphoma is an aggressive form of non-Hodgkin lymphoma involving B cells. It occurs as the result of chromosome translocation involving the Myc gene. It was first described by Dr. Denis Burkitt in 1958 while he was working in Uganda. Burkitt's lymphoma was later discovered to be highly associated with the Epstein-Barr virus; this was the first time a virus was linked to a form of cancer. (Jonathan, *et al*. 2000).

1.1.3. Nasopharyngeal carcinoma:

Nasopharyngeal carcinoma (NPC) is a tumor arising from the epithelial cells that cover the surface of the nasopharynx. NPC was first described as a separate entity by Regaud in 1921 (Regaud. 1921) . Approximately one third of nasopharyngeal carcinomas are of undifferentiated type diagnosed in adolescents or young adults. (Young and Miller. 1975). Although NPC is a rare malignancy throughout most of the world, (Parkin, 2002), .it is endemic in a few well-defined populations. In 2002, 80,000 incident cases of nasopharyngeal cancer were diagnosed worldwide and the estimated number of deaths exceeded 50,000, making it the 23rd most common new cancer in the world; in contrast, NPC was the fourth most common new malignancy in Hong Kong (Parkin , *et al*. 2002) . Arising in the epithelial lining of the nasopharynx, The WHO classified NPC into three histologic types: keratinizing squamous cell carcinoma (type I); and nonkeratinizing carcinoma, characterized as differentiated (type II) or undifferentiated Type III NPC comprises over 95% of NPC in high-incidence

areas, and most of the remaining 5% is type II NPC (Zong, *et al*, 1983;Yu and Henderson. 1996). In almost all populations surveyed, the incidence of NPC is 2- to 3fold higher in males than in females (Parkin, *et al* .2002).

1.2. Justifications

According to the World Health Organization (WHO), roughly 20% of all cancers worldwide results from chronic infections; in particular, up to 15% of human cancers is characterized by a viral etiology with higher incidence in developing countries. The link between viruses and cancer was one of the pivotal discoveries in cancer research during the past Century. Indeed, the infectious nature of specific tumors has important implications in terms of their prevention, diagnosis, and therapy. In the 21st Century, the research on viral oncology field continues to be vigorous, with new significant and original studies on viral oncogenes and translational research from basic virology to treatment of cancer. In Sudan there is increase in the frequency of cancers generally and especially lymphoma and nasopharyngeal carcinomas, with little studies concerning the underline causative agents. This study was investigating the association of Epstein- Barr virus in lymphoma and nasopharyngeal carcinoma

1.3. Objectives

1.3.1. General objective:

- ◊ To study the association of Epstein - Barr virus (EBV) and lymphomas and nasopharyngeal carcinomas among Sudanese patients.

1.3.2. Specific objectives:

- ◊ To detect Epstein - Barr virus in different types of lymphomas by using PCR and Immunohistochemistry.
- ◊ To investigate the association of Epstein - Barr virus and different types of nasopharyngeal carcinomas.
- ◊ To correlate between Epstein - Barr virus infection and demographics characteristic of patients.

2. Literature Review

2.1. The nature of lymph:

Lymph is derived from interstitial fluid and originates in the interstitial spaces of most of the body's tissues. A vast system of converging lymphatic vessels funnels lymph to the thorax where it is returned to the circulation via the thoracic duct. When foreign antigens invade the body, antigenic material, antigen presenting cells known as dendritic cells (*DCs*) and inflammatory mediators generated by local immunological activity at the site of infection are all picked up by the lymphatic vessels and swept along in the flow of lymph. The system of lymphatic vessels has been called an "information superhighway" because lymph contains a wealth of information about local inflammatory conditions in upstream drainage fields (von Andrian and Mempel .2003).

At many sites along the lymphatic highways where lymphatic vessels converge, lymph flows through soft, pale tan, rather lumpy looking lymph nodes that contain large numbers of lymphocytes, macrophages and antigen presenting cells (*APCs*) (Tilney .1971). Mice have 22 identifiable lymph nodes (Van den Broeck, *et al*., 2006) while humans have about 450 . Inside the lymph nodes, (*APCs*) and naive lymphocytes are brought together to initiate primary immune responses (Kaldjian, *et al* .2001); (*APCs*) display antigens to lymphocytes, reactive lymphocytes undergo clonal expansion to produce new lymphocytes and plasma cells, and the resulting plasma cells secrete antibodies into the lymph. These immunological processes take place in a specialized stromal structure called the reticular meshwork that supports guides and organizes interactions between lymphocytes and (*APCs*) (Gretz, *et al* .1997).

Lymph nodes consist of multiple lymphoid lobules surrounded by lymph-filled sinuses and enclosed by a capsule. The complex three dimensional lobules and their surrounding sinuses present a variety of appearances in tissue sections depending on the plane of section (Sainte-Marie *et al*., 1990).

2.2. Lymph node structure (lymphoid Lobule):

The lymphoid lobule is the basic anatomical and functional unit of the lymph node. The smallest lymph nodes may contain only a few lobules or even just one, while large lymph nodes may contain a great many , lobules were described in lymph nodes as early as 1975 (Kelly .1975). Although some authors have described them as physiological compartments (Belisle and Sainte-Marie, 1990) .

Lobules are anchored in the hilus by their vascular roots but they are otherwise separated from the capsule by the subcapsular sinus. The apex forms part of the nodal cortex and the base forms part of the nodal medulla. The nodal cortex is bilayered and consists of a superficial cortex and a deep cortex. By common convention, pathologists usually apply the term cortex to the superficial cortex and refer to the deep cortex as the paracortex. The (superficial) cortex contains spherical follicles that are surrounded and separated by inter follicular (or diffuse) cortex. The paracortex consists of deep cortical units (*DCUs*). Each lobule has a single (*DCU*) that can be anatomically and functionally divided into a central (*DCU*) and a surrounding peripheral (*DCU*) of adjacent lobules often fuse into large multiunit complexes .Sub compartmentalization of the lobule creates separate areas for T and B cells to interact with their (*APCs*) and to undergo clonal expansion. B lymphocytes home to primary follicles to survey follicular dendritic cells (*FDCs*). Stimulated B cells proliferate within the follicles forming distinctive germinal centers and the follicles are then referred to as secondary follicles. T lymphocytes home to the paracortex and interfollicular cortex to survey DCs. Stimulated T lymphocytes proliferate in the paracortex and enlarge it but do not produce structures analogous to germinal centers. The peripheral *(DCU)* and the interfollicular cortex also serve as transit corridors for lymphocytes migrating to and from the B and T cell areas. Plasma cell precursors produced by B cell proliferation migrate to the medullary cords where they mature and secrete antibodies that are released into the lymph. Each lobule

is surrounded by a complex system of lymphatic sinuses that are divided into subcapsular, transverse and medullary sinuses. In large animals, lymph nodes with trabeculae also have trabecular sinuses. A single afferent lymphatic vessel delivers a constant stream of lymph to the sub-capsular sinus over each lobule. Lymph spreads through the subcapsular sinus over the lobule's apex, flows down the sides of the lobule through transverse sinuses and then flows into the medullary sinuses. Lymph from all the lobules drains into a single efferent lymphatic vessel that exits the node at the hilus. Because each afferent lymphatic collects lymph from a different drainage field, each lobule is potentially exposed to a different set of antigens, (*APCs) and* inflammatory mediators . As a result of varying immunological stimulation, lobules within the same lymph node may have different levels of immunological activity and the cortical, paracortical and medullary compartments composed of these lobules will not necessarily have a uniform appearance (Sainte-Marie, *et al* .1982).

2.2.1. Reticular Meshwork:

The reticular meshwork is a delicate, porous, sponge-like tissue composed of stellate; spindle shaped or elongated fibroblastic reticular cells (*FRCs*) and their reticular fibers. The entire lymph node is filled with reticular meshwork. It forms the framework of the lobules and it criss crosses the lumens of the sinuses. The lobular reticular meshwork is composed of stellate (*FRCs*) whose processes subdivide the lobule into innumerable narrow channels and interstices that are occupied by lymphocytes, macrophages and (*APCs.*) (*FRCs*)have large, irregularly oval nuclei and pale cytoplasm and their cell bodies and larger processes can be seen in between and amongst the more basophilic lymphocytes that fill the channels created by their processes. The interstices are 10 to 20 microns wide, wide enough to allow lymphocytes to move through them freely but narrow enough that all the lymphocytes can remain in contact with the reticular cells (Kaldjian, *et al* . 2001). The surfaces of (*FRCs*) are coated with migration ligands, such as fibronectin, that facilitate lymphocyte adhesion and

amoeboid migration (Ruco *et al*.1992; Gretz *et al*., 1996; Kaldjian, *et al*. 2001). Lymphocytes move through the interstices by adhering to and crawling along the huge surface area of (*FRCs*) which serves as highways for the migrating cells (Gretz, *et al*.1996; Kaldjian, *et al*. 2001). At the periphery of a lobule, lobular *(FRCs)* flattens out to form a layer that encloses and defines the lobule and separates it from the surrounding sinuses. The lobule has been described as a labyrinthine space or chamber because of the complexity of the channels formed by the reticular meshwork (Kaldjian, *et al*. 2001).

The reticular meshwork that spans the sinuses has thinner, more delicate branches and correspondingly larger interstices than the lobular reticular meshwork (Luk, *et al*.1973). They tend to occur in clusters, especially in transverse sinuses near the capsule, and are relatively infrequent in the subcapsular sinus (Sainte-Marie, *et al*.1982). Sinus histiocytes increase in response to the need for particle clearance and may completely fill the sinuses (sinus histiocytosis) . Some sinus histiocytes originate in the tissues and migrate to the sinuses after antigenic stimulation (Grande *et al*., 1990). Mast cells in the peripheral tissues can also migrate to the sinuses in response to certain hypersensitivity conditions and express chemokines that regulate T cell recruitment (Tedla *et al*., 1998). The sinuses are lined by a layer of flattened (*FRCs*). The interface between a lobule and a sinus is a trilaminar membrane formed by the layer of flattened sinusal (*FRCs*), the layer of flattened lobular FRCs and a layer of basement membrane or basal lamina sandwiched between them (Moe 1963; Farr *et al*., 1980; Kaldjian *et al*., 2001). This thin membrane can be difficult to appreciate by light microscopy, but it prevents lymph, cells and particulates from passively entering the lobules (Anderson and Anderson, 1975; Sainte-Marie *et al*., 1982; Gretz *et al*., 1996). Dendritic cells (*DCs*) do actively penetrate this barrier, (*FRCs*) have some characteristics of epithelial cells. They can form flattened sheets that are morphologically indistinguishable from lymphatic endothelium (Ushiki *et al.*, 1995). They express cytokeratins 8

and 18 (Franke and Moll, 1987) and form tight junctions with each other. FRCs are also have characteristics of fibroblasts. They secrete slender strands of extracellular matrix known as reticular fibers. Reticular fibers are composed of a core of collagen fibrils enveloped in a layer of basement membrane (Forkert, *et al* .1977). Components that have been identified in reticular fibers include collagens type I, III and IV, elastin, entactin, fibronectin, laminin-1, tenascin, vitronectin, and heparan sulfate (Sainte-Marie and Peng, 1986; Gretz, *et al*., 1996; Kaldjian, *et al.*, 2001).

2.2.2. Cords of lymph node:

The relationship between the reticular meshwork, the lobular blood vessels and the sinuses is fundamentally important to lymph node function and is most easily appreciated in the medulla. Branching medullary arterioles arise from the hilar artery and radiate centrifugally, and condensing medullary venules return centripetally to the hilar vein. A pair of arterioles and venules may run along the central axis of a cylindrical sheath of reticular meshwork surrounded by a dense network of capillaries (Okada, *et al*. 2002),. All the vessels are suspended in the meshwork by pericytic FRCs (Anderson *et al*., 1976; Gretz, *et al*., 1996, 1997; Crivellato and Mallardi. 1997; Okada, *et al.*, 2002). The interstices of the meshwork are filled with recirculating lymphocytes. These perivascular lymphocyte sheaths or cords are the basic repeating unit of the lobule (Gretz, *et al*., 1997; Kelly, 1975). In the medulla they are called medullary cords. In the peripheral DCU they have been termed paracortical cords (Kelly, 1975).

2.3. Lymphoma: Risk Factors:

The cause of the majority of lymphoma cases is unknown. However, several factors may influence risk of developing lymphoma. The relative effects of these factors in any given case of cancer are variable and very difficult to determine with accuracy at this time. Some of these risk factors are

2.3.1. Sex:

Specific subtypes of non-Hodgkin lymphoma, such as follicular lymphoma, are predominant in women; however, non-Hodgkin lymphoma is overall more common in men. Mantle cell lymphoma shows the highest predisposition in males (70% of cases are men) (Ekstrom-Smedby .2006).

2.3.2. Geography:

Non-Hodgkin lymphoma is most common in developed regions of the world, specifically the United States, Australia, New Zealand and Europe, (Ekstrom-Smedby .2006). Epstein Barr virus (*EBV*), a type of herpes virus that infects B lymphocytes, increases a person's risk of developing fast growing lymphomas. In Africa and Southeast Asia, EBV is related to the development of Burkitt lymphoma and Hodgkin's lymphoma, (Learn about Lymphoma, 2010).

2.3.3. Genetics:

Mutations can cause cancer by enhancing cell division and/or reducing tumor suppressor mechanisms. Lymphoma is rarely caused by inherited mutations in the (*DNA*) sequence and there is no increased risk of lymphoma in children of lymphoma patients. (Learn About Lymphoma, 2010).

2.3.4. Age:

The incidence of lymphoma had peaks over 70 years of age, (Grulich and Vajdic. 2005). Non-Hodgkin lymphoma is rarely observed in children and most commonly develops in adults. Less than 1% of non-Hodgkin lymphoma diagnoses reported in 2001 occurred in children under the age of 15 years, (Grulich, Vajdic. 2005). The age groups most frequently affected by Hodgkin's lymphoma are early adults (age 15-40) and late adults (above 55) (Learn about Lymphoma. 2010).

2.3.5. Medical History:

One's medical history may influence their susceptibility to the development of lymphoma. .Individuals with autoimmune diseases had an increased risk of developing lymphoma. Examples of diseases associated with

risk in development of lymphoma include: Diabetes type 1 and Rheumatoid Arthritis, (Landgren, *et al*. 2005)**.** Immunosuppressive therapies used to encourage acceptance of transplanted organs may also increase risk of lymphoma, (Grulich and Vajdic. 2005).

2.3.6. Infections:

Infection with certain viruses and bacteria are associated with increased risk of developing lymphoma. These include:

- **Human immunodeficiency virus (*HIV*)**: this virus is the causative agent of AIDS. The relative risk of non-Hodgkin lymphoma is increased 60–200 fold in HIV-infected patients when compared with the general population (Biggar, *et al.* 1994; Rabkin. 1994)For certain subtypes of lymphoma, notably primary central nervous system (CNS) lymphoma, the risk for HIV-infected patients was increased 1000-fold over the general population during the early years of the AIDS epidemic (Berel, *et al*. 1991)
- **Epstein-Barr virus (*EBV*)**: infection with (*EBV*) is associated with increased risk of lymphoma. In certain geographic regions, including Africa, infection with EBV is associated with Burkitt's lymphoma(Takada. 1999; Chow. 1993)
- **Hepatitis C virus (*HCV*)**: Fisher and colleague said that several pathogens have been linked to the risk of lymphoma, including Epstein-Barr virus, human immunodeficiency virus, hepatitis virus, (Fisher and Fisher. 2004). Hepatitis viruses especially for hepatitis C and hepatitis G have been mentioned as a risk factor for development of Hodgkin's lymphoma (Takada. 1999; Chow. 1993)
- **Human T-cell leukemia virus type 1(*HTLV-1*)**: World Health Organization (WHO) classification of hemato-lymphoid neoplasms (2008) has attributed the genesis of adult T-Cell leukemia/Lymphoma (ATLL) to a human retrovirus, the HTLV - 1, which is endemic in South Western Japan, the

Caribbean basin and parts of Central Africa, and very rare in India(Oshima, *et al.*2008; Bhargava and Dalal. 2008)

- ***Helicobacter pylori* (*H. pylori*)**: This bacterium infects the stomach and is thought to cause ulcers. Infection is also associated with an increased risk of lymphomas of the stomach, (National Cancer Institute Website 2010, Grulich, and Vajdic. 2005).

2.4. Anatomy and histology of nasopharynx:

The nasopharynx (post-nasal space) is the part of the pharynx posterior to the nasal cavities and above the level of the junction of the hard and soft palate. The nasopharynx has a roof, floor and walls on its posterior and lateral sides .It is a trapezoidal chamber posterior to the nasal choanae and superior to the soft palate.(Bailey. 1998). At birth, the nasopharyngeal epithelium is composed primarily of pseudo stratified columnar epithelium that lines most of the upper airway. At on year age, areas of this epithelium are replaced increasingly by stratified squamous epithelium so that beyond age 10 years, most of nasopharyngeal mucosa ,with exception of that overlying the adenoid pad , has been replaced with stratified squamous epithelium .Some authors suggest that this change is not intrinsically programmed genetically , but it represents a metaplastic reaction to environmental insults . Along the lateral nasopharynx wall, small patches of pseudo stratified columnar epithelium remain, intermixed with stratified squamous mucosa ;in the regions of the nasopharynx where these two epithelium types meet , a third type, transitional or intermediate epithelium , resides. Transitional epithelium contains globular or cubical cells are named because of its microscopic resemblance to transitional epithelium of the bladder. The nasopharyngeal mucosa membrane also contains elements of lymphoid tissue and minor salivary glands, which are important considerations in the differential diagnosis of a mass in these locations (Charles, et al. 2005; Tom and Jacobs.2002).

2.5. Definition of nasopharyngeal carcinoma:

Nasopharyngeal carcinoma (NPC) is a tumor arising from the epithelial cells that cover the surface and line the nasopharynx. NPC was first described as a separate entity by Regaud and Schmincke in 1921(Regaud .1921; Schmincke. 1921) approximately one third of nasopharyngeal carcinomas of the undifferentiated type are diagnosed in adolescents or young adults. Although rare, NPC accounts for one third of childhood nasopharyngeal neoplasms (data from USA) (Young and Miller.1975)

2.6. NPC: Epidemiology and incidence worldwide:

Nasopharyngeal carcinoma (*NPC*) has a remarkably distinctive ethnic and geographic distribution. In the year 2000, a total of 64,798 new cases were registered worldwide, and more than 80% of those were reported from China, Southeast Asia, and some Asian countries (Ferlay, *et al* . 2001*).(NPC*) is a rare malignant disease, with an incidence well under 1 per 100,000 persons per year in Caucasians from North America and other Western countries. In contrast, the highest incidence is found among Southern Chinese (25–30 per 100,000 persons per year), especially those of Cantonese origin. Southern Chinese immigrants also have a higher risk of (*NPC*) as compared to the local Western population. Early onset of NPC is observed in high-risk populations. Independent of race/ethnicity, men are 2- to 3-fold more frequently affected than women (Yu and Yuan. 2002). The dramatic difference in the incidence among populations and geographic areas suggests a strong association of (*NPC*) with genetic and environmental factors. An unusually early-age onset in the high-risk populations implies that early events in life may be important. There are recent changes in the epidemiology as shown by decreasing incidences of (*NPC*) (30%) in Hong Kong over the past 20 years (Lee, *et al*. 2003), which may be related to changes in environmental factors.

2.7. Epidemiology of Nasopharyngeal carcinoma in Sudan:

Nasopharyngeal carcinoma (*NPC*) is frequent in the Sudan, accounting for 5.8% (n=374) of all cancer cases reported from the (*SCR*) and for 7.2% (n=512) of those reported from Radio Isotope Centre Khartoum (*RICK*) , these are among the highest (*NPC*) frequencies in cancer series worldwide with male/female ratio of 3:1. (*NPC*) was the commonest tumor in males at (*RICK*) (12.1%) and the second commonest in the Sudan Cancer Registry (*SCR*) (9.2%). It seems to occur in young patients (the youngest reported case was 3-year-old), with 14% and 12.1% of the cases within 14 years of age in the (*SCR*) and (*RICK*) series respectively (Hidayatalla, *et al*. 1983). However, a lower frequency of NPC in children has been reported recently at (NCI-UG), (National cancer Institute), where between 1999 and 2007, (NPC) accounted for about 3.4% of all childhood cancers (Abuidris, *et al*. 2008) geographical distribution of the treated NPC cases suggests that racial susceptibility could play a significant role, while environmental factors were not identified; however, as elsewhere, Epstein –Barr virus is most likely associated with this malignancy also in the Sudan (Hidayatalla, *et al*. 1983). A report describes the (*NPC*) patients treated at (*NCI-UG*) during the period 2000 – 2005. The total number of patients was 103. Age ranged from 11 to 82 years, with a median of 41 years, and a mean of 45.5 years. Male: female ratio was 2:1. The *(WHO*) histology type-3 accounted for 73.8% of cases, (*WHO*) type-2 for 26.2%, and no WHO type-1 cases were found in the series. Neck swelling was the most common presenting symptom. This pattern of NPC presentation resembles that seen in other endemic areas, such as South China. Identifying risk factors for (*NPC*) in the Sudan is a research priority (Abuidris, *et al*. 2008).

2.8. A world Health Organization; classification of nasopharyngeal cancer:

(*NPC*) is a distinctive type of head and neck cancer. The World Health Organization (*WHO*) classification distinguishes three histopathological types of NPC based on the degree of differentiation. Type I is keratinizing squamous cell

carcinoma (*SCC*), similar to other head and neck cancer .Type II is nonkeratinizing carcinoma, and Type III is undifferentiated carcinoma (Shanmugaratnam and Sobin, 1991). The undifferentiated carcinoma has a typical morphology with a prominent lymphoplasmacytic infiltrate, and is also referred as "lymphoepithelioma." Different prevalent histologic types of (*NPC*) are found in endemic and nonendemic regions. In endemic areas such as Southern China, (*WHO*) Type III accounts for more than 97%, while keratinizing SCC is more common in the Western countries (75%). (Marks, *et al*. 1998). Aside from differences in histological features, latent Epstein-Barr virus (EBV) infection is uniquely present in almost all, if not all, (*NPC*) from endemic regions, but absent in (*WHO*) Type I (*NPC*) from nonendemic regions). Alternative pathogenic processes of EBV negative NPC, especially (*WHO*) Type I from Western populations, may be involved. (Raab -Traub. 2002)

2.9. Etiologies of nasopharyngeal carcinoma:

The ethnic clustering of (*NPC*) in Southern Chinese strongly suggests the involvement of genetic susceptibility and environmental factors in its development. Three major etiological factors including genetic factors, environmental, and viral infections are illustrated here.

2.9.1. Genetic factors:

Early linkage analysis on Chinese sib pair studies of (*NPC*) suggested the association of susceptibility (*HLA*) haplotypes with NPC development. The investigators hypothesized that some of the (*HLA*) antigens have reduced efficiency in activating host immune response to (*EBV*) infection, which plays a critical role in the pathogenesis of (*NPC*). Most studies conducted among Chinese showed that individuals with HLA-A2 are at increased risk. A recent high-resolution genotyping study has detected a consistent association between (*NPC*) and the prevalent Chinese A2 subtype (HLA-A0207), but not the prevalent Caucasian subtype (HLA-A0201) (Hildesheim, *et al*. 2002). Supported by affected sib pair haplotype sharing analysis and association study on (*HLA*)

regions, a (*NPC*) susceptible gene/locus closely linked to the MHC region but distinct from the (*HLA*) genes was proposed (Lu, *et al*.1990). However, recent linkage analysis of Chinese NPC pedigrees using highly polymorphic microsatellite markers further identified two susceptibility loci on chromosomes 4p15.1-q12 and 3p21, respectively, but not on *(MHC*)region, from the Guangdong and Hunan provinces in China (Feng, *et al*. 2002; Xiong *et al*., 2004). Polymorphisms of genes for carcinogen metabolism (CYP2E1), detoxification (GSTM1), and (*DNA*) repair (XRCC1 and hOGG1) were also reported to be associated with increased risk of (*NPC*) (Hildesheim, *et al*. 1997; Nazar-Stewart, *et al* .1999; Cho, *et al* .2003).

2.9.2. Environmental factors:

The traditional foods of Southern Chinese, such as Cantonese style salted fish and other preserved foods containing volatile nitrosamines, are an important carcinogenic factor for (*NPC*) .The childhood consumption of salted fish has been shown to be related to an increased risk of (*NPC*) in Southern Chinese (Yu and Yuan. 2002). In animal studies, nasal and nasopharyngeal tumors could be induced in rats by feeding them Chinese salted fish (Huang, *et al*.1978). These traditional diets may act as chemical carcinogens that induce genetic damage in nasopharyngeal epithelial cells.

2.9.3. Epstein-Barr virus (EBV):

In contrast to other head and neck cancer and epithelial malignancy in general, nasopharyngeal carcinoma has a unique feature of and it has strong association with (*EBV*). Higher EBV antibody titers, especially of IgA class, are observed in most (*NPC*) patients. Latent (*EBV*) infection is identified in cancer cells of virtually all cases of NPC in endemic regions. The clonal (*EBV*) genome is consistently detected in invasive carcinomas and high-grade dysplastic lesions (Raab-Traub and Flynn, 1986; Raab-Traub. 2002).

2.10. Epstein Barr Virus (Taxonomy, structure, and biology):

2.10.1. Taxonomy

The Epstein-Barr virus (*EBV*), the first isolated human tumor virus, was identified in 1964 by Epstein's group in a cell line derived from Burkitt lymphoma (Epstein, *et al.* 1964). (*EBV*) is a human herpesvirus, classified within the gamma herpes virus subfamily, and is the prototype of the *Lympho crypto virus* genus. In keeping with the systematic nomenclature adopted for all human herpes viruses, the formal designation of (*EBV*) is human herpesvirus 4 (HHV-4). Two major EBV types have been detected in humans: EBV-1 and EBV-2 (also known as types A and B). EBV-1 and EBV-2 differ in the sequence of the genes that code for the EBV nuclear antigens (EBNA-2, EBNA-3A/3, EBNA-3B/4, and EBNA-3C/6) (Sample, *et al.* 1990). EBV-2 immortalizes B cells less efficiently than EBV-1 *in vitro*, and the viability of EBV-2-infected lymphoblastoid cell lines is less than that of EBV-1-infected lines (Rickinson, *et al.*1987). The differences in the immortalizing efficiency of the EBV subtypes may relate to a divergence in the (*EBNA-2*) sequences (Cohen.*et al.* 1989). In addition to type-specific polymorphism, significant DNA-sequence heterogeneity has been found when comparing selected regions of the EBV genome isolated in certain geographic areas or even from the same area. These polymorphisms define different viral strains within both types (Aitken, *et al.* 1994).

2.10.2. Target cells:

Like other gamma herpes viruses, (*EBV*) establishes latent infection in lymphocytes and can induce proliferation of the latently infected cells (Young and Rickinson. 2004). (*EBV*) infection of B cells is mediated through the interaction of the viral envelope glycoprotein gp350/220 with the cellular receptor for the C3d complement component CR2 (CD21) (Fingeroth *et al.*, 1984, 1988; Tanner, *et al.*1987). After binding of the viral particle to the surface of the host cell and endocytosis, the viral envelope fuses with the host-cell

membrane by a mechanism involving three other viral glycoproteins: gp85, gp25, and gp42 (Li. 1995). It is worth noting that gp42 can bind to major histocompatibility complex (*MHC*) class II, and (*EBV*) uses this as a cofactor in the infection of B lymphocytes (Li, *et al.* 1997). It has been shown nonetheless that EBV can also infect cells, albeit at low efficiency, via CD21- independent mechanisms. Indeed, cells that do not express CD21 (as some epithelial cells) can be infected by the virus, and furthermore a virus deficient in gp350/220 was shown to be still infectious (Imai, *et al.* 1998; Janz, *et al.* 2000). Although(*EBV*) is considered to be a B-lymphotropic virus, it can also infect T lymphocytes or epithelial cells because it is found in some T-lymphoma cells and several important diseases of epithelial cells, including nasopharyngeal and gastric carcinomas, and oral hairy leukoplakia (Thompson and Kurzrock. 2004). Other CD21-independent pathways may be responsible for (*EBV*) infection of cells, other than B lymphocytes (Imai, *et al.* 1998; Janz, *et al.* 2000). Current evidence suggests that (*EBV*) infection in healthy chronic virus carriers is largely restricted to B cells, although in certain situations the virus can be detected in epithelial cells. The most likely role for epithelial cells is as a site for replication and amplification of (*EBV*) rather than as a site of persistent latent infection, however, this remains controversial (Kieff. 1996).

2.11. Viral gene expression:

The expression of (*EBV*)-encoded proteins differs depending on the type, differentiation, and activation status of the infected cell. The growth-stimulating program is based on the expression of six nuclear and three membrane proteins. Six of these are essential for the activating and proliferation-driving effect of the virus. One virally encoded nuclear protein, EBNA-1 which is required for the maintenance of the viral episomes, is expressed to various degrees in these cells (Thorley-Lawson. 2005). In all forms of latency, EBV expresses two classes of non-coding small RNA (*EBER*) 1 and2, which are highly structured RNAs of 167 and172 nucleotides, respectively. The expression of (*EBER-1*) and -2 is

restricted to the cell nucleus where they are present at approximately 107copies per cell (Sample. 2008). Also ,EBV encodes at least 22 micro-RNAs which are expressed to various degrees in latency I, II, III(Cai, *et al.* 2006; Grundhoff, *et al.* 2006).Specific latency EBV-transcription programmes have been demonstrated in many human tumours, including immunoblastic lymphoma in immunosuppressed patients ,Burkitt lymphoma, Hodgkin disease, and nasopharyngeal carcinoma . The origins of all of these tumours can be understood as arising from specific stages in the EBV life cycle, and appear to be associated with disturbances of the immune system a (Thorley and Lawson. 2005). Latency I is generally associated with the EBV-related Burkitt lymphoma, latency II with Hodgkin disease, T-cell non-Hodgkin lymphoma, and nasopharyngeal and gastric carcinoma; latency III occurs mainly in immunocompromised individuals, in post transplant lymphoproliferative disorders, and HIV-associated lymphoproliferative disorders (Liebowitz, &Kieff. 1993; Sbih-Lammali, *et al.* 1996; Niedobitek, *et al.* 1997; Cesarman and Mesri. 1999; Kis, *et al.* 2006 ; Klein, *et al.* 2007).

2.12. Epidemiology of infection:

2.12.1. Prevalence, geographic distribution:

Many epidemiological studies have shown that EBV is highly prevalent throughout the world (IARC. 1997;Young. 2008), Although primary EBV infection during early childhood is usually subclinical or has symptoms that are similar to other respiratory illnesses, a delay in acquiring a primary EBV infection at an older age in childhood or adolescence ,which usually occurs in more developed countries (Rickinson and Kieff. 1996),.In a study in the Hong Kong Special Administrative Region (Chan, *et al.* 2001),sequential measurements for markers of(*EBV*) infection from serum samples of a group of infants demonstrated a sudden seroconversion at the age of 8 months, which may imply a protective role for persistent maternal antibodies, and also partially explain why primary (*EBV*) infection in early childhood, unlike during

adolescence, is usually asymptomatic (Chan, *et al.* 2001).Two major types of EBV – EBV-1 and EBV-2– have been identified and differ in geographic distribution. The role of specific EBV types in the etiology of different cancers is unknown .Immunocompromised patients more commonly harbor both subtypes of EBV (Borisch, *et al.* 1992; Thompson and Kurzrock. 2004). EBV-2 maybe more common in Africa, (Gratama & Ernberg, 1995), and in homosexual men (van Baarle, *et al.* 2000; Higgins, *et al.*,2007). It has been hypothesized that the attenuated transforming ability of EBV-2 along with an immune suppressive condition (HIV or malaria) may be necessary for EBV-2 to be capable of maintaining infection of B lymphocytes, and to cause transformation (Buisson, *et al.* 1994; Thompson and Kurzrock. 2004). However, other studies showing that HIV-infected haemophiliacs have lower rates of EBV-2 infection than HIV-infected homo sexual have challenged this hypothesis, and suggest that the acquisition of EBV-1 versus EBV-2 would rather be due to the opportunity for exposure(van Baarle, *et al.* 2000; Thompson and Kurzrock. 2004).The fact that EBV is ubiquitous, and consequently causes widespread and largely asymptomatic infection, suggests that the specific geographic distribution of EBV-associated malignancies ,such as endemic Burkitt lymphoma and nasopharyngeal carcinoma, is probably not due to differences in EBV infection but rather due to the activation of viral replication by additional cofactors (Young. 2008).

2.12.2. Transmission and risk factors for infection:

EBV infection usually occurs in individuals of a young age, with low socioeconomic status or development, from a larger than average family, and with poor hygienic standards. By their third decade of life, 80–100% of these individuals become carriers of the infection (IARC. 1997).The oral route is the primary route of transmission of the virus; however, transmission by transfusion has been documented. In developing countries, infection is acquired in the first few years of life. Crowding and/or the practice of pre-chewing food for infants

may be contributing factors. In the developed world, infection is often delayed to adolescence, when transmissions more likely because of intimate oral exposure (Hjalgrim, *et al.* 2007). About 50% of primary (*EBV*) infections during young adulthood result in clinical infectious mononucleosis (CDC. 2006).Infectious mononucleosis is usually acquired from a transfer of saliva, and in young adults, this is more likely to occur after the onset of sexual activity. However, only limited data are available to support this hypothesis (Macsween and Crawford. 2003). In a cohort study of sexually active young women, the development of detectable antibodies against EBV after primary infection increased with increasing number of sexual partners, and was greatest when a new sexual partner was encountered in the 2 years before seroconversion. In addition, transient EBV DNA loads were detected in cervical cytology samples in some of the women (Woodman, *et al.* 2005).

2.12.3. Persistency, latency, and natural history of infection:

Following primary infection via transmission of cell-free virus and/or of productively infected cells in saliva, (*EBV*) will enter into the circulating B-cell pool, and then remain in most cases undetected for life in a latent state (Young and Rickinson, 2004; Thorley Lawson. 2005). (*EBV*) can also infect the mucosal epithelial cells in which intermittent viral productive replication occurs (Frangou, *et al.* 2005). The B-cell compartment, more precisely resting memory B cells, appears to be the true reservoir of the latent virus in healthy carriers. Resting memory B cells express a very restricted pattern of latent viral gene expression (Miyashita, *et al.* 1995); this is how these infected cells can persist in the face of efficient cytotoxic T lymphocyte (*CTL*) surveillance (Masucci and Ernberg. 1994). Nonetheless, cells that express the full repertoire of growth transformation-associated antigens are likely be generated sporadically in asymptomatic virus carriers, because memory *(CTLs*) that are reactive against most (*EBV*) are maintained at high levels for life (*Monograph*; *(IARC*. 1997).Primary (*EBV*) infections occurring in adolescence or early in adult life

are manifested as infectious mononucleosis, which is an acute form of primary infection occurring asymptomatically in early childhood. (*EBV*)-associated malignancies are suspected to result from viral reactivation that is most likely due to interaction with additional cofactors (Young. 2008).

2.13. Biological markers of the different status of EBV infection:

2.13.1. Antibody responses to EBV:

The detection of antibodies to (*EBV*) in biological fluids has been until recently the major means of diagnosis for EBV infection. Distinct patterns of antibody response have been identified during primary infection, latent infection of healthy carriers, viral reactivation, and in various (*EBV*)-associated diseases. Serological parameters include the detection of IgG, IgM, and occasionally IgA, directed against (*EBNAs*), early antigens (*EAs*, divided into two components, EA-D (encoded by *BMRF-1*) and EA-R (a human BCL-2homologue encoded by *BHRF-1*)), and *VCAs* (IARC. 1997).

2.14. Infectious mononucleosis:

Most information available on primary antibody response has been provided by studies on infectious mononucleosis. .At the onset of clinical symptoms of the disease, substantial titer of IgM antibodies to *(VCA*) are detected, with rising titer of IgG to EA, and to *(VCA*). IgA antibodies to these antigens may also appear. Whereas anti-VCA IgM titers disappear over the next few months, anti-(*VCA*) IgG titers rise to a peak that may fall slightly, and anti-EA IgG titers become either undetectable or stabilize at very low levels. Neutralizing antibodies to the major envelop glycoprotein gp350 are detected during the acute phase of infectious mononucleosis but only at very low titers, and increase to stable levels thereafter (IARC. 1997).The serology of infectious mononucleosis for the (*anti-EBNA*) response presents an interesting pattern. For the (anti-*EBNA*) response, during the acute phase of infectious mononucleosis, patients show an IgG response to *EBNA*-2 (and also probably to *EBNA*-3A, -3B, and -3C), whereas an IgG response to *(EBNA*-1) is not usually detected until

convalescence. The production of antibodies to (*EBNA*-1) and (*EBNA*-2) in the course of infectious mononucleosis follows an ordered progression (Henle, *et al.*1987; IARC. 1997).

2.15. Healthy EBV-carriers:

IgG antibodies to (*VCA)*, to neutralizing antigp350, and to *(EBNA*-1) are consistently detected in the serum of healthy carriers. The titer of these antibodies is usually stable over time but can markedly differ among individuals (Henle and Henle. 1976). Antibodies to (*EA*) are only detected in a proportion of healthy carriers. Although persisting for life, (anti-*EBNA*-1) and (anti-*VCA*) do not appear to have much of a protective role (Moss, *et al.* 1992).Virus shed can be frequently detected from throat washes of asymptomatic carriers. The levels of shedding are thought to be quite stable over many months, although with different rate depending on the individuals. A direct relationship appears to exist between the level of virus shedding in the throat and the level of virus-infected B cells in the blood. However, no obvious relationship was shown between the levels of (*EBV*) virus shedding from the throat and either (anti-*VCA*) or (anti-*EA*) titers in the serum of healthy carriers (Yao, *et al.* 1985).

2.16. EBV and Carcinogenesis:

2.16.1. Establishment of Persistent Infection:

The mechanism of tumor causation by (*EBV*), depends on the tumor type. However, certain basic general characteristics of (*EBV*) biology are found in the various (*EBV*)-associated cancers. The first stage in the mechanisms of (*EBV*) tumourigenesis is the establishment of a persistent infection (Kutok and Wang. 2006). Persistent infections occur in greater than 95% of adults. The relatively low incidence of (*EBV*) related tumours compared with this prevalence of infection shows that there are definitely many other factors that contribute to tumor development. The primary infection with (*EBV*) is believed to start within the oropharyngeal epithelial cells with viruses subsequently passing to sub epithelial B cells through direct contact. The invasion of the immune system by

(*EBV*) infection of B cells stimulates a vigorous CD8+ T cell response. The development of a virus-specific adaptive immune response is largely responsible for the elimination, to a large extent; of (*EBV*) infection by reducing the number of (*EBV*) infected B cells. This elimination is however incomplete. Persistent (*EBV*) infection remains as a latent infection in peripheral blood lymphocytes and as a lytic infection in the oral cavity, which results in the shedding of infectious viruses *via* oral secretions (Kutok and Wang. 2006). (*EBV*) infects resting B cells and turns them into continuously proliferating lymphoblastoid cell lines that express nine latency-associated viral proteins, including six nuclear antigens (Epstein-Barr nuclear antigen (*EBNA*)-1, - 2, -3A, -3B, -3C and -LP) and three membrane proteins (latent membrane protein (LMP)- 1, -2A and -2B) (Gruhne, *et al*. 2009).

However, only some of these proteins are expressed in *EBV*-carrying malignancies. Genomic instability is a hallmark of malignant transformation and is frequently associated with chromosomal aberrations, including reciprocal translocations, deletions, inversions and duplications, which deregulate the expression of oncogenes or tumor suppressor genes (Raptis and Bapat. 2006). These aberrations are clonally transmitted and constitute useful cytogenetic markers of malignancy. In addition, nonclonal chromosome aberrations, such as dicentric chromosomes, chromosome fragments, gaps, double minutes and rings, are generated by inappropriate repair of (*DNA*) breaks. These aberrations are usually lethal to dividing cells and are therefore generated de novo during each cell cycle. Several studies have reported the presence of clonal chromosome translocations in (*EBV*) associated malignancies (Stollmann, *et al*. 1985; Klein. 1986). In their publication,(Gruhne, *et al. 2009)* showed the specific roles of three (*EBV*) latency proteins (Stollmann, *et al.* 1985). They demonstrated that the (*EBV*) nuclear antigens, *EBNA*-1 and *EBNA*-3C, and the latent membrane protein 1, *LMP*-1, independently promote genomic instability, as detected by nonclonal chromosomal aberrations, (*DNA*) breaks and phosphorylation of histone *H2AX*.

EBNA-1 promotes the generation of (*DNA*) damage by inducing reactive oxygen species (*ROS*), whereas *(DNA*) repair is inhibited in *LMP*-1-expressing cells through down regulation of the *(DNA)* damage-sensing kinase, ataxia telangiectasia mutated (*ATM*), reduction of phosphorylation of its downstream targets Chk2 and inactivation of the G2 Check point. *(EBNA*-3C) enhances the propagation of damaged *(DNA*) through inactivation of the mitotic spindle checkpoint and transcriptional down regulation of BubR1. Thus, multiple cellular functions involved in the maintenance of genome integrity seem to be independently targeted by (*EBV*), pointing to the induction of genomic instability as a critical event in viral oncogenesis. Additionally, in several cancer cells types, the (*EBV*) genome is heavily methylated and few viral genes are expressed. Methylation of *DNA* is usually associated with inhibition of gene expression; partly mediated by the association of specific methyl-Cp G-binding proteins with methylated (*DNA*), leading to transcriptional silencing and chromatin remodeling; and also by the inhibition of *(DNA*) binding of some transcription factors through (*DNA*) methylation (Karlsson, *et al*. 2008**)**.

2.16.2. Transformation of Cells in Vitro:

When peripheral blood lymphocytes from chronic virus carriers are placed in culture, the few (*EBV*)-infected B cells that are present regularly give rise to spontaneous outgrowth of EBV-transformed cell lines, known as lymphoblastoid cell lines (*LCLs*), provided that immune T cells are either removed or inhibited. These cells all carry multiple copies of the viral episome. *EBNA*-2 and *LMP*-1 have been demonstrated to be absolute requirements for this *in vitro* transformation while *EBNA* leader protein, *EBNA*-3A, *EBNA*-3C, and *LMP* 2A play important roles in the process (Kieff**.** 1996).

2.17. EBV- associated malignancies in immunocompromised individuals:

2.17.1. Tumor Formation in Immune Suppressed Individuals:

EBV infected B cells grow into (*LCLs) in vitro* when cultured in the absence of T cells. This implies that the prevention of spontaneous generation of B cell tumors from infected B cells is related to the intact immune system. It follows therefore that B cell tumors will develop in conditions of severe immune suppression. This has been demonstrated experimentally in animal models in cotton top tamarins and in severe combined immunodeficient (*SCID*) mice. In these cases the proliferation can be oligoclonal or monoclonal and they all resemble *(LCLs)* latency III pattern of (*EBV*) gene expression and in cell surface phenotype .The obvious inference from the remarkable efficiency of tumor development in these experimental models is that the development of immunoblastic lymphomas in severely immunosuppressed humans only requires (*EBV*) induced B cell transformation, with no necessity for secondary genetic or epigenetic changes. It is also notable that tumor regression usually follows reduction in the level of immunosuppression if the cancer is therapy induced. These particular lesions are therefore quite different in pathogenesis from other (*EBV*) positive malignancies, where viral infection is but one event in a complex multistep process of cancer development. Three main types of lymphomas are seen in different categories of immunocompromised persons: Lymphomas in individuals with inherited immunodeficiency states referred to as X-linked lymphoproliferative disorders , Lymphomas associated with iatrogenic immunosuppressant therapy given to transplant recipients, and (*AIDS*) associated lymphoproliferative disorders(Young, *et al*. 2000**) .**

2.17.2. Post-Transplant Lymphoproliferative Disorders (PTLDs):

It is now well established that there is a high incidence of lymphoproliferative disorders following solid organ and hematopoietic stem cell transplantation (*HSCT*. *PTLDs*) are made up of a variety of almost exclusively B cell proliferations ranging from spontaneously regressing, polytypic

lymphoplasmacytoid B cell expansions which resemble inflammatory reactions to lethal clonal B cell proliferations typically resembling non-Hodgkin's lymphomas (*NHLs*). Most *(PTLDs*) are associated with (*EBV*) infection. However, this association is not strong in *(PTLDs*) that occur very late after transplantation (Kutok and Wang. 2006). The World Health Organization (*WHO*) has classified these disorders into early-onset *(PTLDs*) and late-onset *(PTLDs*) (Borish, *et al*. 2001). Early-onset *(PTLDs*) are usually the (*EBV*) driven polyclonal lymphoproliferative disorders while the late-onset (*PTLDs*) include the true monoclonal diseases such as polymorphic (*PTLD)* and monomorphic *(PTLD*); the latter being further sub classified into Burkitt's /Burkitt's-like lymphoma, diffuse large B-cell lymphoma (*DLBCL*), and classical Hodgkin's lymphoma (*cHL*) (Carbone, *et al*. 2008). Specifically, WHO has sub classified these lymphoid proliferations into 4 main categories reactive, plasmacytic hyperplasias or infectious mononucleosis-like lymphoid hyperplasias, polymorphic (*PTLD)* and monomorphic *PTLD* corresponding to B and T cell (rare) (*NHL*), and (*H)L* and *HL*-like *(PTLD)* (rare). It is however very difficult to make any distinctions between these lymphoid proliferations. A typical characteristic of these tumours is their extra nodal multifocal manifestations. The degree and duration of immunosuppressive therapy has been found to be directly related to the incidence of *(PTLD)* either in solid-organ or hematopoietic stem cell transplantation. The incidence of (*PTLDs*) following solid organ transplantations has been found to be relatively higher than following *(HSCT*), probably due to more prolonged and intensive immunosuppressant therapy in solid organ recipients. Frequencies of *(PTLDs*) have ranged from 1% in renal transplant patients to about 3%-8% in heart and lung transplant recipients. Following *(HSCT, PTLD)s* occur in about 1% of patients (Zutter, et *al*. 1988).

2.17.3. HIV/AIDS-Associated Lymphomas:

HIV/*AIDS*-associated lymphomas constitute a heterogeneous group of lymphoproliferative disorders arising in the setting of profound immunosuppression in the terminal phases of (*HIV*) infection. This severe immunosuppression allows uncontrolled proliferation of (*EBV*) infected lymphocytes. This group of lymphomas includes primary central nervous system (*CNS*) lymphomas, diffuse large B cell lymphomas (*DLBCL*), Hodgkin's lymphoma (*HL*), Burkitt's lymphoma (*BL*) or BL-like lymphomas, and primary effusion lymphomas. Variations exist in the frequency of *(EBV*) infection in AIDS-related lymphomas depending on the subtype of tumor involved. *(CNS*0 lymphomas in *(AIDS*) patients have been mostly affected with EBV infection. One potential explanation for this finding is that *HIV*-induced immunodeficiency may increase the trafficking of *EBV*-infected B cells into the brain, or the combined immunodeficiency and immune privileged nature of the *(CNS*) favors (*EBV*)-driven B lymphoproliferations (Kutok and Wang. 2006). Most cases of (*DLBCL*) with immunoblastic morphology and *(HL*) in *(AIDS*) patients are (*EBV*) related. These cases appear in the final stage of *(AIDS*) when the patient is severely immunocompromised. In contrast, only 30%-50% of cases of Burkitt or Burkitt-like lymphomas in *(AIDS*) patients are associated with (*EBV*) infection (Raphael, *et al*. 1994; Davi, *et al* 1998; Diebold, *et al*. 1997), in these cases, (*EBV*) infection and immunosuppression may increase the pool of B cells at risk for a *c-MYC* translocation and may not be the primary mechanism for driving malignant proliferation. (*EBV*) infection can be detected in some primary effusion lymphomas, but these B cell lymphoproliferations are linked consistently to *(KSHV*) infection, which suggests that the gamma-2 herpesvirus is more important for this malignancy than (*EBV*) infection (Kutok and Wang. 2006).

2.18. Burkitt's lymphoma:

Burkitt's lymphoma (*BL*) is an aggressive B cell tumor. The different types include the endemic type, the sporadic type and the immunodeficiency associated type. Whatever the type, the fundamental transforming event in (*BL*) is the translocation of the (*MYC*) gene. This translocation is to one of 3 chromosomes from the normal position on chromosome 8. The 3 destination chromosomes are 14 (to the region of the Ig heavy chain), 2 (to the region of the Ig kappa genes) and 22 (to the region of the Ig lambda genes). In 80% of cases the t(8;14)(q24;q32) translocation occurs with the (*IGH*) gene. The remaining 20% of cases are split between the translocations with the *(IGK)* and *(IGL)* (corresponding to t(2;8) and t(8;22) respectively) (Brady, *et al*. 2008). the effect of the reciprocal translocation is to create a constitutively activated *(MYC)* gene. The (*MYC)* proto-oncogene plays a critical role in regulating cell proliferation, differentiation and apoptosis depending on the type of cell or other situations (Eisenman and Deconstructing. 2001). In (*BL)*, the outcome of the (*MYC)* mutation can be summarized as cell growth, uncontrolled proliferation, and increased number of subsequent mutations occurring in the genome. Not all (*BL*) are (*EBV*)-associated but the chromosomal translocations remain fairly constant, differing only in the breakage points on the Ig gene. It is clear that (*EBV*) does not act in (*BL*) as a direct transforming virus but through an indirect manner. Its role appears to be in stimulating B cell proliferations thereby increasing the risk of spontaneous translocations in the B cell line. It has also been shown to increase the rate of somatic hyper mutations due to virus induced extended expression of the enzyme activation induced deaminase (*AID*). (*EBV*) also acts by obstructing the apoptotic pathways that are normally activated when there is excessive (*MYC)* activity. In endemic (*BL*), it acts in concert with other factors like malaria which seem to act by reducing T cell response to (*EBV*) antigens through a non *EBV*-specific activity. The events that bring about this translocation and those that allow cells to survive with the constitutive

expression of (*MYC)* has been the subject of intense investigations. (*EBV*) infection, malaria, immunodeficiency and spontaneous somatic mutations all contribute to the origin and maintenance of this cancer (Diebold, *et al*. 1997).It is also important to note that the inactivation of two common tumor suppressor genes (*TSGs*), the cyclin dependent kinase inhibitors (p16/CDK4A and p15/CDK4B), through promoter hyper methylation, and the inactivation of the p53 homologue p73 are frequently observed in BL. Further, the epigenetic inactivation of members of the (*BCL2*)-family or other pro-apoptotic gene families is a common event in (*BL*). A couple of broad-spectrum *(TSGs*) and genes involved in cell cycle control, apoptosis, intra cellular signaling, proliferation, and surface adhesion are also frequently methylated in (*BLs*) (Niller, *et al*. 2009). (*BL*), first described by Dr .Dennis Burkitt, in 1958, was initially thought to be a sarcoma of the jaws (Burkitt, 1958). Subsequently however, further studies revealed that this tumor was a unique type of non-Hodgkin's lymphoma with a peculiar epidemiology and a very strong association with (*EBV*) (Orem, *et al*. 2007; Brady, *et al*. 2008). The disease originally described is the Endemic (*BL*) which is largely found in Africa. It typically affects the facial skeleton, particularly the jaw bones, in children between 2 and 9 years .Endemic (*BL*) occurs frequently (in 50-100 cases per 1,000,000 individuals) in the equatorial regions of Africa and Papua, New Guinea. Most cases of endemic (*BL*) are associated with the presence of (*EBV*) in the tumor cells (Kutok and Wang. 2006).The earliest descriptions of the geographical distribution of (*BL*) coincided with the so-called lymphoma belt of Africa which was associated with malaria hyper- and holo endemicity, (Wright, *et al*. 1971). This lead to the hypothesis that malaria or some other infectious agent carried by mosquitoes was responsible for the tumor. The roles of malaria in (*BL*) causation are not fully understood. The most common hypothesis in the relationship is the profound immunosuppression caused by malaria. Malaria suppresses T-cell responses including those directed against (*EBV*) (Ho, *et al*. 1988).Sporadic

Burkitt's lymphoma was described outside the African region, but it is morphologically similar to endemic (*BL*). It predominantly affects the organs of the abdominal cavity. Sporadic (*BL*) can be found in any age group and has not been associated with any specific co-factor. The incidence of sporadic *(BL*) is much lower than the endemic disease, with 2-3 cases per 1,000,000 individuals in the equatorial regions of Africa and Papua, New Guinea. In the United States and Europe the association with (*EBV*) is low (15-30% of cases) (Brady, *et al.* 2008;Hecht and Aster. 2000; Gutierrez, *et al*. 1992). *(HIV)*-associated BL is the third variant that was subsequently described in the era of *(HIV*) infection. Although it has been commonly reported in the developed world and associated with (*HIV*) in some adults in Africa, the childhood variety of the disease among (*HIV*) infected children has not been well characterized. However, it can be seen in any geographical location and in all age groups (Brady, *et al.* 2008; Diebold, *et al.*2001; Parkin, *et al*. 2003).

2.18.1 EBV and Burkitt's lymphoma:

The initial restricted African geographical distribution of (*BL*) was intriguing prompting studies searching for the possible viral cause, eventually leading to the discovery of the novel human herpesvirus. EBV is a lymphotrophic gamma human Herpesvirus, the first virus to be associated with a human tumor (Thorley. 2008). In review of (Thorley-Lawson, *et al. 2008;* Hecht and Aster. 2000), described the curious relationship between (*EBV*) infection and Burkitt's lymphoma. The most compelling argument for a direct role for (*EBV*) in (*BL*) pathogenesis was the initial discovery that almost 100% of (*BL*) in equatorial Africa was associated with (*EBV*) infection. It was also shown that children who were infected early in life and who produced the highest antibody titres to the virus were at highest risk for developing the tumor. The tumor cells were found to express (*EBNA*), a serologically defined, tumor antigen, in their nuclei, although this antigen was later shown to be composed of six components, of which only one, (*EBNA-1*), was expressed in (*EBV*)-positive (*BL*) (Hecht and

Aster. 2000; Wright. 1967). Subsequently, (*EBV*) was firmly linked to other proliferative diseases, including acute infectious mononucleosis, nasopharyngeal carcinoma ,Hodgkin's disease immunoblastic lymphoma in individuals who are immunosuppressed, a subset of gastric carcinomas ,rare T- and NK-cell lymphomas and leiomyosarcoma (Hecht and Aster . 2000 ; Deyrup. 2008). The surprising result that Epstein-Barr virus (*EBV*) was not restricted to (*BL*) patients as initially believed, but was not only associated with various other cancers but also found to be widespread with prevalence in about 95% of the adult population made it an even more exciting subject of research. One observation that favored the carcinogenic role of (*EBV*) in (*BL*) was the finding that (*EBV*) was a potent transforming virus in culture for the same cell type that develops into (*BL*), the B lymphocyte (Hecht and Aster. 2000). (*EBV*) appears to be an extremely efficient transforming virus in culture, being able to convert >50% of all target cells (the resting B cell) into continuously proliferating, latently infected lymphoblastoid cell lines (*LCLs*) within a few days. In spite of the very close association between (*EBV*) and BL, the precise roles of (*EBV*) in (*BL*) tumourigenesis remained difficult to understand for a very long time. This was because, although (*EBV*) has been detected in virtually all cases of endemic (*BL*), most cases of sporadic and (*HIV*) related (*BL*) were not (*EBV*) associated. Further studies therefore led to the understanding that (*EBV*) is a cofactor in the pathogenesis of (*BL*) (Hecht and Aster. 2000).

2.19. Hodgkin's Lymphoma:

Hodgkin's lymphoma (*HL*) has been referred to as an unusual neoplasm due to the fact that the malignant cells constitute only a minority of cells in the tumor mass. Classical HL (*cHL*) is characterized by the presence of clonal, malignant multinucleated Reed Sternberg giant cells in a background of reactive inflammatory cells that includes lymphocytes, plasma cells, granulocytes, and histiocytes .Most of the proliferations within these tissues result from there active inflammatory cells that accompany the Reed Stenberg cells. The Reed

Sternberg cells typically constitute less than 1% of the cellularity. The presence of the Reed Sternberg cells is pathognomonic for this tumor. (*HL*) exists in 5 forms: mixed-cellularity, nodular-sclerosis, lymphocyte rich, lymphocyte-depleted and lymphocyte-predominant subtypes. All of these subtypes apart from the lymphocyte predominant HL (*LPHL*) constitutes what are now known as the classical HL (*cHL*). (*LPHL*) is a unique clinicopathological entity not associated with (*EBV*). Classical HL (*cHL*) is a complex and multifactorial disease. (*EBV*) infection is associated with approximately40% of (*cHL*) cases most frequently with the mixed-cellularity subtype. This subtype is more common in males and demonstrates a bimodal age distribution (<10 and >50 years of age). (*EBV*)-negative (*cHL*) is most common in adolescents and young adults, does not show a gender predisposition, and is associated with a high standard of living in childhood (Kutok and Wang. 2006). It was in 1966 that MacMahon first proposed that an infectious agent might be a possible agent in the pathogenesis of (*HL*) (MacMahon. 1966). This was supported by subsequent findings in which raised antibody titres to (*EBV*) antigens were found more in (*HL*) compared with other lymphoma patients (Levine, *et al*. 1971) The fact that the detection of raised levels of (*EBV*) preceded the development of (*HL*) by several years also supported the growing suspicion that (*EBV*) contributed to the carcinogesis in (*HL*) (Mueller, *et al*. 1989) Another significant contribution was the study by Gutensohn, *et al*. in which it was reported that, following infectious mononucleosis, the relative risk of developing (*HL*) compared to individuals without prior history ranged between 2.0 and 5.0 (Gutensohn and Cole . 1980). The first report of the successful demonstration of (*EBV DNA*) in (*HL*) tissue specimens was by Weiss *et al.* in 1991 using the cloned *Bam* HI W fragment of EBV, as an *in situ* hybridization probe (Weiss, *et al*. 1991).When present, EBV is clonal, which indicates clonal expansion of single (*EBV*)-infected cells. In this cells (*EBV*) infection exhibits a type II form of latency, (*EBV*) gene expression being limited to the (*EBERs*), Epstein-Barr nuclear antigen 1 (*EBNA1*), latent

membrane protein 1 (LMP-1) in high levels, LMP2, and the Bam HIA transcripts (Deacon , *et al*. 1993).*In situ* hybridization probes were developed to target highly abundant (*EBERs*) which provided a reliable and simple method for the detection of (*EBV*) in archival (*HL*) specimens (Wu, *et al*. 1990) Initial studies showed that (*EBV DNA*) was detected in 20-25% of (*HL*) tumor specimens (Wu , *et al*. 200). The detection of EBV DNA by *in situ* hybridization provided the first demonstration of its existence in the Reed Stenberg giant cells (Langdon, *et al*. 1986; Anagnostopoulos, *et al*. 1989). Subsequently, the demonstration of the abundant (*EBV*) early RNA (*EBER-1 and EBER-2*) sequences in Reed Stenberg cells provided a sensitive method for detecting latent infection in situ. This technique is generally accepted as the "gold standard" for the detection of latent (*EBV*) infection in clinical samples (Langdon, *et al*. 1986).The mechanism of transformation in (*HL*) appears also to be indirect. The malignant cells of (*HL*) are believed to derive from the germinal centre B cell (Kuppers and Rajewsky. 1998). These cells are those that have acquired serious Ig mutations resulting in nonfunctional Igs. These types of damaged cells would under normal circumstances have undergone apoptotic elimination .This apoptosis is overcome if there is a high level of activation of NF kB. The EBV LMP-1 is a potent activator of (*NFB*), and the activation of (*NFB*) is an important step for EBV-induced B cell immortalization. In addition, the (*EBV -LMP-2A*) can mimic signaling effectively through the Ig receptor (Caldwell , *et al*. 1998).Reed Stenberg cells exhibit a type II form of latency ,EBV gene expression being limited to the (*EBERs, EBNA-1*), LMP-164 LMP-2, and the Bam HIA transcripts (Klein . 1983).It has been variously reported that several epidemiological factors such as sex, age, ethnicity, country of residence and histological subtype have roles to play in the association between (*EBV*) and HL. In particular, in developed populations, the association between (*EBV*) and (*HL*) is less, with percentages of between 20% and 50% for North American and European cases (Klein . 1983;Herbst, *et al*. 1992 ; Hummel , *et al*. 1992) 57% for Chin abut

much higher rates in underdeveloped countries such as Peru (Chang, *et al*. 1993) and Kenya (Jarrett , *et al*. 1996; Weinreb , *et al*. 1996 ; Weinreb , *et al*. 1996).It has been postulated that the increased incidence of (*EBV*) positive (*HL*) in underdeveloped countries is a consequence of the existence of an underlying immunosuppression similar to that seen in African (*BL*) in a malaria infected population (Wu, *et al*. 1990). In spite of the increased knowledge about the contributory roles of the various carcinogenic mechanisms in (*HL*), the precise contribution of (*EBV*) remains yet to be fully understood. In particular, it is very important to identify the roles of latent virus products, particularly (*LMP-1*) and (*LMP-2*). A number of (*TSGs*) involved in cell cycle control, apoptosis, and surface adhesion was frequently silenced by methylation in (*HL*) tumours: p16 and p15 were methylated at higher frequency in relapsed tumors than in primary tumours. Studies have shown that the pro apoptotic (*CHK2*) kinase was sporadically silenced in various carcinomas and lymphomas, but was completely silenced in (*HD*) cell lines. Contrary to (*BL*), the "death associated protein kinase" (*DAPK*) was methylated at a low frequency, and mainly at advanced tumor stages. Like in (*BL*) cells, PCDH10 was highly methylated in (*HD*) cell lines. Impaired Ig production in RS cells despite their mostly functional gene rearrangements has also been attributed to probable epigenetic closedown of the Ig H promoter (Niller, *et al*. 2009).

2.20. T/NK nasal type lymphoma:

Apart from B cells which are known to be primarily infected by (*EBV*), other non-B-cell (*NHLs*) associated with (*EBV*) infection include T-cell lymphoproliferative disorders which include a subset of peripheral T-cell lymphomas such as extra nodal nasal type NK/T-cell lymphoma. Several unique genotypic and phenotypic features have been identified in this subset of (*EBV*) associated lymphomas, some of which are absence of T-cell antigens, the expression of the (*NK*) cell marker CD56, and the absence of T-cell receptor gene rearrangement. These tumors typically occur in the nasal and upper

aerodigestive area. (*EBV*) infection has been found to be consistently associated with these lymphomas, irrespective of geographical location (Kieff. 1996; Baumforth, *et al*. 2000).

2.21. Epithelial malignancies:

EBV has been demonstrated in many epithelial malignancies, the most consistent one and arguably the most important in this regard is the undifferentiated (nonkeratinizing) carcinoma of the nasopharynx, which is also called lymphoepithelioma because of the profuse lymphocytic infiltration that accompany the tumor cells .Here the association is such that regardless of geography early 100% of the tumors and all the tumor cells have been demonstrated to be monoclonally (*EBV*)-positive. Lymphoepithelioma-type tumors occurs in many organs ,and (*EBV*) has been shown to be positively associated with a large number of these tumors, including those in the salivary gland, stomach, middle ear, lungs, and thymus (Crawford. 2001;Leung, *et al*. 1995).

2.21.1. Nasopharyngeal Carcinoma (NPC):

Nasopharyngeal carcinoma has been reported in almost all parts of the world, however, most cases are found in South East Asia, Southern China (including Hong Kong),North Africa and in the Eskimo population of Alaska, US (Gullo, *et al*. 2008 ; Ballinger, Li . 2000 ; Cohen. 2000 ; Huang, *et al*. 1993).The incidence reaches a peak of around 20-30 cases per 100,000 with rates being highest in individuals of Chinese descent irrespective of where they live, and particularly in Cantonese males. It has been found out that ckitis highly expressed in the juvenile form of North African nasopharyngeal carcinomas with a significant association between (*LMP-1*) and c-kit expression (Charfi, *et al*. 2007). There is no doubt that (*NPC*) has a definite association with the (*EBV*) (Niedobitek. 2000). The persisting controversy, however, is the specific pathogenic mechanism by which (*EBV*) causes (*NPC*). This is in spite of the fact that virtually all cases of nonkeratinizing (*NPC*) are (*EBV*) positive in all

geographical locations. Specifically, the direct role of (*EBV*) in the carcinogenesis of (*NPC*) is strongly disputed .One of the problems confronting the explanation of the (*EBV*) in the pathogenesis of the tumor is the fact that mature nasopharyngeal cells are not usually infected with (*EBV*) *in vivo* and *in vitro*. Yet the tumors have been shown to be infected with (*EBV*) before transformation. It has been shown that the immature epithelial cells carry CD21 and can be infected by the virus. It is therefore postulated that (*EBV*) infects nasopharyngeal cells that have been stimulated by other environmental factors. The area of emphasis appears to be the importance of dietary carcinogens, such as salted fish products (Jarrett, *et al*. 1996; Weinreb, *et al.* 1996). Salted fish and preserved food top the list of dietary agents implicated in (*NPC*) (Yu. 1992). Areas that have been found to be mostly affected are southern China, Tunisiaand Greenland, where the diet contains large amounts of salted fish and preserved food rich in N-nitro sodium ethyamine (*NDMA*), N nitrospyrrolidene *(NPYR*) and Nnitrospiperidine (*NPIP*) (Jarrett, *et al.* 1996; Poirier, *et al*. 1987). Exposure to smoke or chemical pollutants, including trace elements (e.g. nickel) are some of the environmental factors which have also been reported to be associated with the development of (*NPC*) (Jarrett, *et al*. 1996; Wu, *et al*. 1990).It appears that latency gene expression in NPC is intermediate between that seen in (*BL*) (latency I program) and (*HL*) (latency II program). The expression of latent viral (*EBNA-1*) and the (*EBER*) genes have been confirmed in all (*EBV*)-positive (*NPC*) cases. It also appears that (*LMP-2A*) may have growth-promoting effects in epithelial cells, and (*LMP-2A*) and (*LMP-2B*) transcripts are amplifiable in most tumors (Kutok and Wang. 2006; Morrison, and Raab-Traub. 2005). However, reports show that (*LMP-2A*) protein can be detected in only about 50% of (*NPC*) (Heussinger, *et al*. 2004). (*LMP-1,mRNA*) is more difficult to detect, and protein is identified readily in only 35% of cases. (*LMP-1*) has been identified in all pre invasive lesions, suggesting that its expression is necessary in early lesions but may not be as essential in established carcinomas

(Pathmanathan, *et al*. 1995). EBV-induced proliferation of epithelial cells may increase the risk of other genetic and epigenetic events that may contribute to tumourigenesis (Kutok and Wang. 2006). Elevated titers of IgA antibody to (*EBV*) viral capsid antigen (*VCA*) are usually found in patients with (*NPC*), therefore this method of measuring patients' EBV-specific IgA antibodies is useful in screening for early detection of (*NPC)* (Weinreb, *et al*. 1996). (*EBNA-1*) and (*EBER*s) are expressed in all (*EBV*) positive cases of (*NPC*) (Niedobitek, *et al*. 1992; Young, *et al*. 1988). (*EBV*) associated (*NPC*) has been proposed as a promising target for virus specific immunotherapy, but this can only be successful after the various controversies regarding the roles of EBV and (*NPC*) carcinogenesis is resolved. Though several epigenetic alterations have been observed in (*NPC*), however (*LMP-1*) and E-cadherin (CDH1) appear to be central to the pathology. (*LMP-1*) transfection into carcinoma cells suppressed CDH1 expression, thereby facilitating a more invasive growth. The CDH1 promoter methylation was found in about 50% of primary tumors, increasing with advanced and invasive tumor stages; and correlating strongly with (*EBV*)-infection. Thus, (*EBV*) appears to contribute to the rapid metastasis, especially when the tumor expresses (*LMP-1*). However, some (*NPCs*) have been known to have low-level (*LMP-1*) expression (Niller and Wolf. 2009).

2.22. Methods of EBV Diagnosis:

The diagnosis of (*EBV*) latency or disease from clinical samples has long been a subject of controversy, largely because (*EBV*) contributes to morbidity and mortality in healthy subjects and particularly in the immunocompromised hosts. The bone marrow and solid organ transplant recipients represent a special class of immunocompromised patients; the former being without immunocompetent specific (*anti-EBV*) lymphocytes for a variable period, while the latter has severely incapacitated lymphocytes, as a direct effect of iatrogenic immunosuppression.

2.22.1. In Situ Hybridization:

The detection of (*EBER*) transcripts by *in situ hybridization* (ISH) is widely regarded as the gold standard and is rightly, the most common method for the molecular diagnosis of EBV-associated malignancies (Gulley , 2001). The *(EBVDNA*) genome can also be detected using probes that recognize its *Bam* HI W internal repeat sequences (Brousset, *et al*.1992) but this is less sensitive. ISH further serves to localize EBV activity to specific cell types within the lesion (Gulley. 2001).

2.22.2. Immunohistochemistry and Immunocytochemistry:

Latent membrane protein-1 (*LMP-1*) immunochemistry has been shown to be just as effective as (*EBER-ISH*) in detecting (*EBV*) in (*PTLD*), Hodgkin's lymphoma, and infectious mononucleosis (Lones, *et al*. 1997). However, it is less useful in non-Hodgkin's lymphoma (*NHL*) or carcinomas. Other immunostain targets include (*EBNA-1, EBNA-2, and LMP-2A*) (Niedobitek, *et al*. 1992).

2.22.3. Viral Nucleic Acid Amplification:

The detection of (*EBV*) nucleic acids can be done using (*PCR*) and nucleic acid sequence-based amplification (*NASBA*); both of which cannot sufficiently differentiate between latency and disease. However, they have found some use in the differential diagnosis of metastatic undifferentiated carcinomas of unknown primary (Shibata, *et al*. 1991) and CNS lymphomas (Antinori , *et al*.1999). On the other hand, quantitative *(PCR*) has been used quite efficiently for *(EBV*) viral load assays; especially in the diagnosis and monitoring of (*PTLD*) and nasopharyngeal carcinoma. The test is sufficiently rapid, sensitive and specific (Bai, *et al*. 1997, Lo, *et al.* 1999).

2.22.4. Serology:

In the immunocompetent host, EBV-specific enzyme link immune sorbet assay (*ELISA*) or immunofluorescent assays can reliably distinguish acute from previous (*EBV*) infection (Svahn, *et al*. 1997). EBV-associated malignancies are

often characterized by markedly high titres against early antigen and IgG viral capsid antigen. (*EBNA*) titres are however reduced, making it non specific for malignancy, and thus inadequate for diagnosis (Niedobitek, *et al*. 1992). On the contrary, nasopharyngeal carcinoma is usually associated with elevated titres against multiple viral antigens, especially IgA antibodies against lytic antigens, reflecting the tumor's mucosal origin (Deng, *et al*. 1995).

2.22.5. Other Methods:

Several other techniques have been used to study the EBV-associated malignancies including Southern blot analysis of (*EBV DNA*), culture of (*EBV*) or EBV-infected lymphocytes and electron microscopy to examine the detailed morphologic changes associated with (*EBV*) infection. Some of these techniques remain relevant even today, for understanding (*EBV*) biology and pathology; but are seldom required in the clinical setting. Newer technologies such as gene expression profiling and array are also being refined to further sub classify and prognosticate these diseases (Niedobitek, *et al*. 1992).

Materials and Methods

3.1. Study Design:

The current study was retrospective and prespective study conducted at the Radio Isotope Center Khartoum (*RICK*), Al-zahrawey Center and Total Lab care Center in Khartoum state -Sudan.

3.2. Study area:

The current study was conducted in Khartoum, which is the capital of Sudan, with its current population of about 5 million. It lies between the White and blue Niles. Khartoum, together with the two cities, Omdurman and Khartoum North constitute the National Capital of the Sudan (tri-capital). Due to continued immigration of people as result of economic hardship and war displacement, the Khartoum population is a fair representation of the general population of the entire Sudan

3.3. Study duration:

The study was conducted during the period from October 2010 to January 2015.

3.4. Study Populations:

The study group comprised Formalin –fixed paraffin embedded Lymph node biopsies of one hundred and thirty five (135) comprised malignant lymphomas and nasopharyngeal carcinomas (case group) as well as control group comprised normal nasopharynx and reactive lymph node hyperplasia , all cases were selected from the surgical pathology obtained from the archives of Radiation Isotope Center Khartoum (*RICK*) after taking the numbers of blocks from the patient records data of the following years (2011, 2012, 2013 and 2014) all the histological samples were confirmed ,by histopathologist .

3.5. Sampling processing:

Two sections of (3-4) μ m in diameters were cut from each block using a rotary microtome. One section was placed on a frosted-end glass slide and stained by (H&E) stain for confirmation of histopathological diagnosis found in

the records and for grading of the tumor. The other section was placed on positively charged slide and dried overnight at58° C for immunohistochemistry. For each paraffin block, 30-50 µm were collected into a screw capped Eppendorf tube by standard collection procedure for polymerase chain reaction (PCR) using new knife and gloves for each block to avoid contaminations.

All quality control measures were adopted during specimen collection and processing, both negative and positive controls supplied by the manufacturer were used.

3.6. Data collection:

The following data was collected from patient's files; personal data, Demographic Data, Analytic Data including (Site of tissue taken biopsy, type of cancer, performed tumor marker).

3.7. Ethical considerations:

The study was approved by ethical committee of the college of graduate studies and scientific Research (Institute Research Board) of the Shendi University, before conducting the study Permission of this study was obtained from the local authorities in the area the Radio Isotope Center Khartoum (*RICK*) Hospital of the study. The aims and the benefits of this study were explained with assurance on confidentiality.

3.8. Data analysis:

The collected data was analyzed by computer, using the statistical programs software Statistical Package for the Social Sciences (SPSS) version (11.5).The following statistical measures were used :-

- Mean , Standard SD, frequency , percentage for quantitative data
- Chi -test and correlation were used for qualitative data (significance level were set at $P \leq 0.05$).
- The data was presented in form of figures and tables.

3.9. Procedure of hematoxylin and eosin stain for paraffin sections:

1. Section was dewxed rehydrated through descending grades of alcohol to water.

2. Section was stained in an alum hematoxylin for 8 minutes.

3. Section was washed well in running tap water ‘blue’ for 5 minutes or less.

4. Section was differentiated in 1% acid alcohol for 10 seconds.

5. Section was washed in tap water until section gained ‘blue’ (10–15 minutes.

6. Section was stained in 1% eosin Y for 10 minutes.

7. Section was washed in running tap water for 1–5 minutes.

8. Section was dehydrated through alcohols, clear, and mounts.

3.10. Immunochistochemical techniques:

Principle:

Identifying cellular or tissue constituents (antigens) by means of antigen – antibody interactions, the site of antibody binding being identified either by direct labeling of the antibody, or by use of a secondary labeling method. (Bancroft, *et al.* 2005)

3.10.1. Immunochistochemical method for nasopharyngeal carcinoma and the controls:

Applications:

Anti-Epstein-Barr virus antibody targets the 60 kDa latent membrane protein (LMP-1) encoded by the BNLF1 gene of the Epstein-Barr virus.

3.10.2. Immunochistochemical method for Lymphoma and their controls:

The Immunochistochemical procedure was done as follows:

Three microns (3µm) sections from formalin-fixed, paraffin-embedded were cut and mounted onto salinized slides (Fisher brand). Monoclonal antibody (LMP- 1) was used as manufacture instructions to detect presence of (*EBV*) from both case and control samples. All sections were deparaffinized in two changes of xylene for 10 minutes in each change, then rehydrated in descending changes of ethanol as follows; sections were placed in two changes of absolute ethanol

for 5 minutes in each change and then were placed in 90% ethanol for 3 minutes, and then were placed in 70% ethanol for 2 minutes, and then were washed in distilled water for 2 minutes. Sections were steamed for antigen retrieval for (*EBV*) using (PT link) in 10mM- citrate buffer Hydrochloric acid (*HCl*) (pH 7.6) for 20 minutes. Then slides were cooled in a sink containing cold tap water, the slides were removed once the buffer at room temperature, slides were rinsed in running tap water and placed in TBS at pH 7.4,. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min, then ultra V block was applied and incubated for 8 min and then Slides were incubated with 100 µl of primary antibodies for 20 min at room temperature in a moisture chamber, and then were rinsed in Phosphate buffer saline. The primary antibody for EBV (Monoclonal antibody (LMP-1) was ready to use (Thermo Fisher). After washing with PBS for 3 min, binding of antibodies was detected by incubating for 15 minutes, with biotinylated goat anti polyvalent followed by incubating for 15 min with streptavidin peroxidase (Thermo Fisher kit). the slides then were be washed with two rinses of TBS pH 7.4 for 1-2 minutes followed , then the slides were be covered by 0.01% H_2O_2 and 0.05 3.3 diamino benzidine tetra hydro chloride (DAB) chromogen in Tris HCl pH 7.6 for 5 minutes at room temperature, (Thermo Fisher) as a chromogen to produce the characteristic brown stain for the visualization of the antibody/enzyme complex . the sections was counter stained with Mayer's Haematoxylin for one minute, washed in tap water for 2 minutes, dehydrated through 3 different baths of absolute alcohol, placed in xylene, mounted in (*DPX*) **.** For each run of staining, positive and negative control slides was also prepared. The positive control slides contain the antigen under investigation and the negative control slides prepared from the same tissue block, but were incubated with PBS instead of the primary antibody. Each slide was evaluated with investigator then the results were confirmed by consultant histopathologist.

3.11. Tissue preparation for polymerase chain reaction (PCR):

For each paraffin block, 30-50 µm were collected into a screw capped Eppendorf tube. To avoid cross contamination, each block was cut with new gloves and new disposable microtome blade

3.11.1. De-paraffinization and re-hydration of sections:

One ml of xylene was add two times to a screw capped Eppendorf containing sections, then the tube was incubated at 37c for 50 minutes, vortexed and centrifuged at 8000g per 5minutes. the pallet was re-hydrated with serial dilutions of absolute ethanol, 75% ethanol,50%ethanol,25%ethanol and sterile water(vortex and centrifuge for 3minutes at maximum speed after each washing and the supernatant was discarded, these steps was followed in each tube

3.11.2. The DNA extraction procedure:

The pellet that was obtained from previous steps treated as the following : 100 µl lysis –Buffer-T, 10 µl of Proteinase K solution was added to a 0.5ml centrifuge tube , then mixed by vortexing ,the sample was added into the buffer , vortexed then incubate at 65°C for 5 min , incubate the sample at 95°C for 3 min, 100 µl of Universal Buffer NST was added , then mixed and vortexed Briefly , the mixture was used as PCR template directly,

3.11.3. EBV PCR:

This PCR targets the EBV gene encoding the viral latent membrane protein one (*LMP-1*) and detects the two types of EBV equally as assessed using the prototype B95-8 and AG876 strains for type 1 and type 2 viruses, respectively (Kieff and Rickinson, 2001; Sample *et al*, 1994). The nucleotide sequences for these PCR primers were 5′ GCGGGTGGAGGGAAAGG-3′ (E3–44mer) and 5′-GTCAGCCAAGGGACGCG-3

3.11.4. PCR protocol:

The PCR reactions were performed in a total volume of 20µl using one-tenth of the extracted DNA in a reaction mixture containing 200 µ mol of each deoxynucleotide triphosphate, 1.5 mM magnesium chloride, 50 mM potassium chloride, 10 m M Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.5 unit of *Thermus aquaticus* DNA polymerase, and 0.2µ M for EBV**.(intron bio tech)** The PCR amplification was performed as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, with an extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

3.11.5. Gel electrophoresis:

The PCR products were visualized in 3% Agarose gel with 0.5 µg/ml Ethidium bromide stain. The gel was prepared by dissolving 3 grams of agarose powder in 100 ml of 1X TBE buffer and heated at 65°C until the agarose completely dissolved then left to cool at RT and 2µl ethidium bromides was added. The comb was placed appropriately in the electrophoresis tray and then gel was slowly poured and left to set for 30 min for solidification. Ten µl of 100 base pairs (bp) DNA ladder and PCR products were loaded on the gel. Gel-electrophoresis was performed at 120V and 36 Am for 40 minutes. Pictures were taken by Gel documentation system (Gel mega, digital camera and software in a computer).

3.11.6. PCR interpretations results:

According to the manufacture. PCR amplification products of the EBV band on ethidium bromide stained agarose gel in size of 400bp indicative of positive LMP-1EBV.

Results

This retrospective and prespective study was conducted in Khartoum state in the period from October 2010 to January 2015, the main aim of this study to investigate the association between EBV infection and lymphoma and nasopharyngeal carcinomas

The study populations was one hundred and thirty five cases (135). (105/135) of them comprised the study group including (lymphoma and nasopharyngeal carcinomas, seventy (52%) (70/135) of malignant lymphomas and thirty five (26%) (35/135) of Nasopharyngeal carcinomas, while thirty (30/135 - 22%), represent control group including non-malignant samples (reactive lymph node hyperplasia and normal nasopharynx tissue) as illustrated in (Figure.4.1).

As regard to the gender distribution of the study population, males represent (68%) (92/135), the highest frequency while females represent (32%) (43/135), as appeared in (Figure. 4.2)

Using Immunohistochemistry technique, positive (LMP-1) of EBV, were reported in thirty four biopsies (32%) (34/105) of malignant lymphomas and Nasopharyngeal carcinomas (case group), compared with four positive(13%) (4/30) biopsies of non malignant tissue (control group), (P.value= 0.04,) , this result showed a significant difference between case and control groups in the association with EBV infection, that was obvious in (Table. 4.1)

Applying polymerase chain reaction (PCR), forty six biopsies were positive (42.8%) (46/105) for EBV DNA among (case group), compared with seven (23.3%) (7/30) EBV positive in (control group), the(P.value= 0.043), this result showed a significant difference between case and control groups concerning the association with EBV infection as illustrated in (Table. 4.2) .

Comparing the association of EBV infection in malignant lymphomas and reactive lymph node hyperplasia, using specific anti Lamp 1 antibody, EBV were detected in(32%) (23/70), and (10%) (2/20) respectively, the (P.value

=0.04), this result showed a significant difference between case and control groups, referring to (Table. 4.3).

(Table.4.4) illustrate that, the positive EBV detected by PCR ,were twenty nine (41.4%) (29/70) of malignant lymphomas, compared with five (25%) (5/20) of lymph node hyperplasia, the (P.value= 0.18), therefore no statistically significant difference between malignant lymphoma and lymph node hyperplasia in the association with EBV infection.

Considering the subtypes of malignant lymphoma, fifty nine (84%) (59/70) were Non Hodgkin` s lymphomas, and eleven (16%) (11/70) as Hodgkin `s lymphomas, as shown in (Figure. 4.3)

In (Figure. 4.4) males were the dominant gender, (73%) (51/70), and female represented (27%) (19/70) of lymphoma cases.

The frequency of malignant lymphomas according to the age groups appeared in (figure 4.5). As the following (47%) (33/70), (30%) (21/70) and (23%) (16/70) , for the age groups(less than 30 yrs), (above 50 yrs) and (30-50 yrs) respectively.

(Table. 4.5) illustrate that EBV positive Immunostain were reported in five (45%) (5/11) of Hodgkin`s lymphomas and (31%) (18/59) of Non Hodgkin`s lymphomas, the (P.value= 0.333), there is no statistically significant difference. Similar results obtained by EBV PCR that were seven (64%) (7/11) of Hodgkin`s lymphomas, and twenty two (37%) (22/59) of Non Hodgkin`s lymphomas, with (P.value.= 0.103), as in (Table. 4.6).

In (Figure 4.6) the frequency of subtypes of Non Hodgkin`s Lymphomas, were (44%) (26/59) for Diffuse large B cell lymphoma, (17%) (10/59) for Burkitt lymphoma,(25.4%) (15/59) for Follicular lymphoma, (3.3%) (2/59), (7%)(4/59)for T cell lymphoma and (3.3%)(2/59)for Marginal zone lymphoma.

The histological types of Non Hodgkin`s lymphomas were affect by age as the (P.value= 0.000). Burkitt lymphoma and Diffuse large B cell lymphoma affected the children and young adults , while Small lymphocytic lymphoma

affected the elderly ,as in (table4.7) , while the gender has no significant association , the (P.value. 0.5), as illustrated by (Table 4.8).

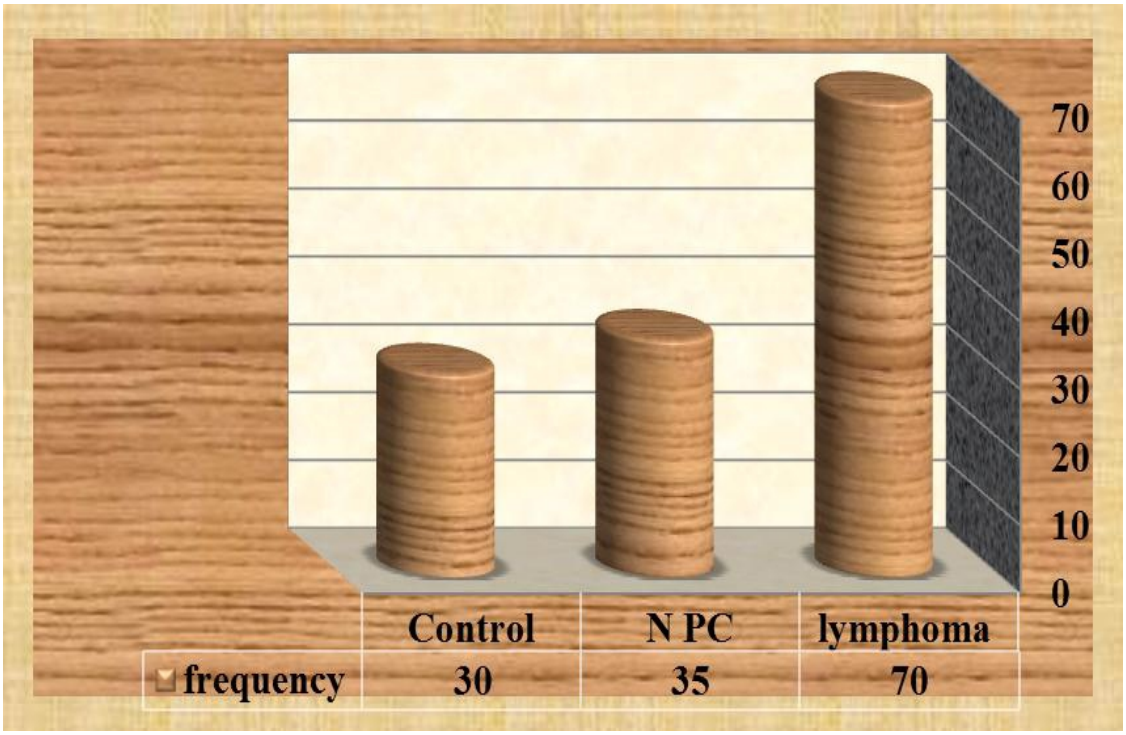
In this study the frequency of nasopharyngeal carcinomas among males and females were (71%) (25/35) and (29%) (10/35) respectively, that is obvious in (Figure 4.7)

(Figure.4.8) Describe the frequency of nasopharyngeal carcinomas among different age groups, It was more frequent in the middle age ,fourteen cases (40%) and fourteen cases (40%) in elderly and less frequent in children and young adults , it was seven cases(7/35 - 20%). .

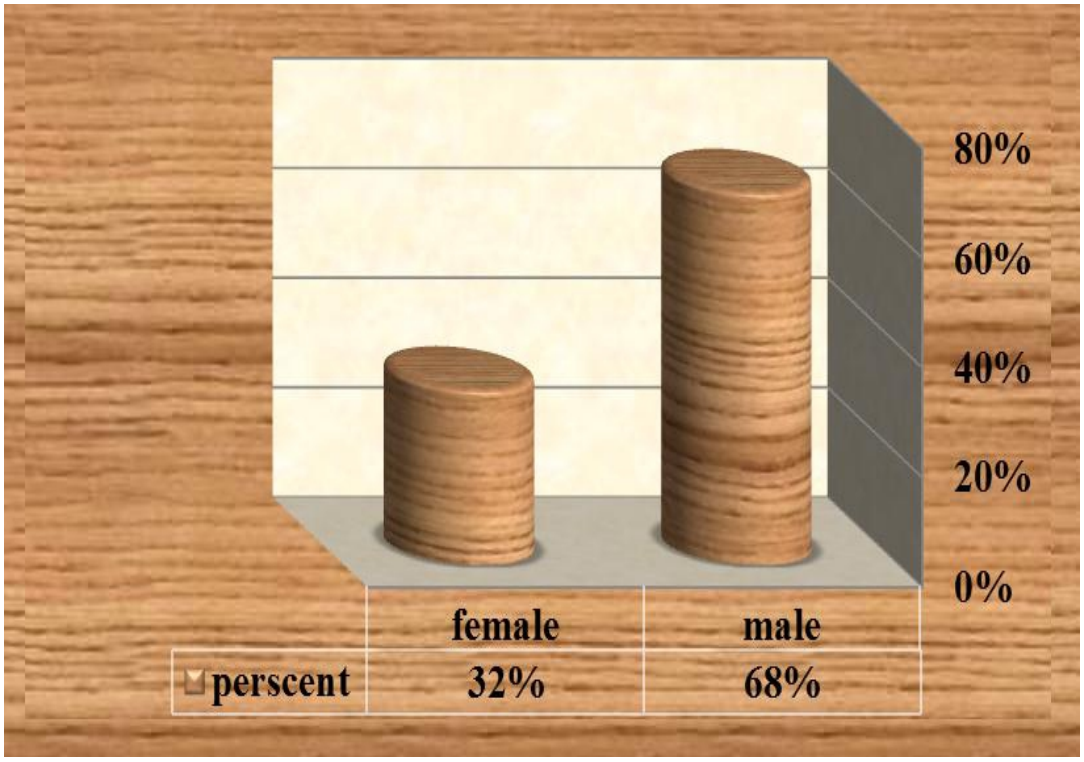
Considering the histological subtypes of nasopharyngeal carcinomas, the highest frequency was Non keratinized Squamous Cell Carcinomas subtypes (54%)(19/35) followed by Undifferentiated Squamous Cell Carcinoma (26%)(9/35), then keratinized Squamous Cell Carcinomas(20%) (7/35), as shown in (Figure.4.9)

(Table.4.9) showed the association of EBV detected by immunohistochemistry (LAMP-1) in the three histological form forms of nasopharyngeal carcinomas, five (55%) (5/9) of undifferentiated squamous cell carcinoma were EBV positive, four (21%) (4/19) of non keratinized squamous cell carcinoma and in two (28%) (2/7) of keratinized squamous cell carcinoma, with (P.value = 0.182), so there is no statistically significant different in the association of EBV with histological subtypes of nasopharyngeal carcinoma.

(Table.4.10) Describe the association of EBV detected by PCR, in the three histological forms of nasopharyngeal carcinomas, EBV were positive in nine (100%) (9/9) of undifferentiated squamous cell carcinoma, five (5/19- 24%) of non keratinized squamous cell carcinoma and in three (43%) (3/7) of keratinized squamous cell carcinoma, the (P.value =0.001), with strong statistically significant different in the association of EBV with histological subtypes of nasopharyngeal carcinoma, as the EBV was detected in all undifferentiated types.



(Figure.4.1): Frequency of the study population



(Figure.4.2): the frequency of sex groups among study population

Table (4:1): EBV Immunostain, comparison between the study group and the control group

Study group	IHC (EBV) result		Total	p. value
	Positive	Negative		
Case	34	71	105	**0.04**
Control	4	26	30	
Total	38	97	135	

(Table.4:2): EBV PCR, comparison between the study group and the control group

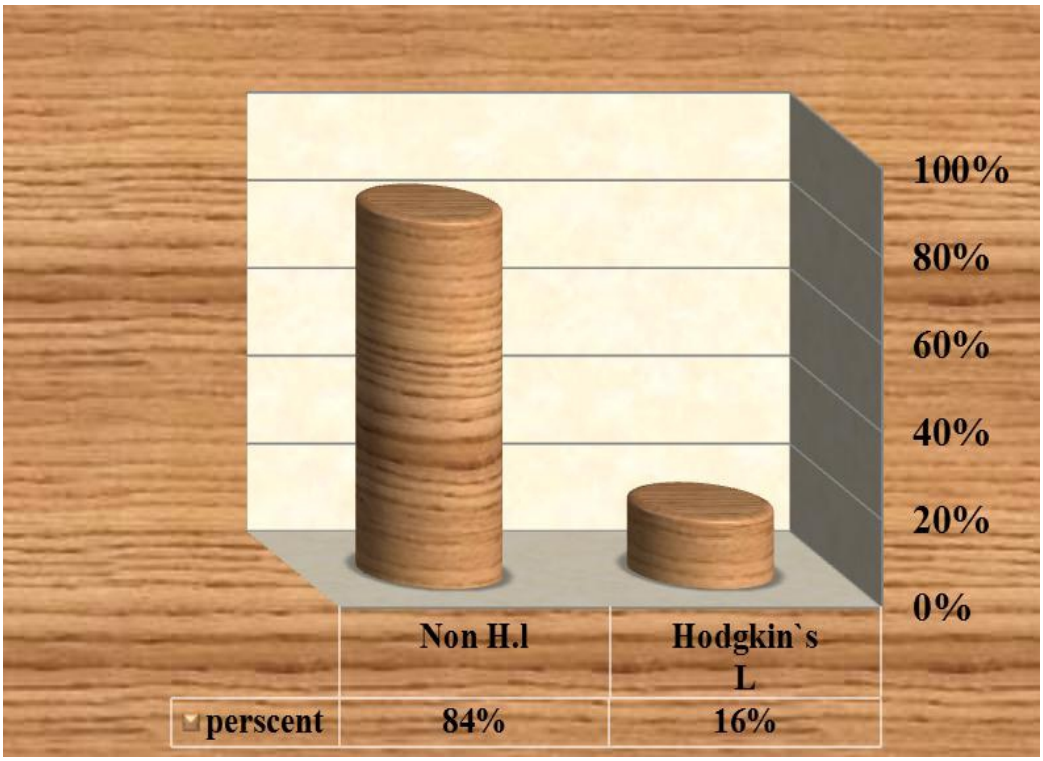
Study group	PCR result		Total	p. value
	Positive	Negative		
Case	46	59	105	**0.043**
Control	7	23	30	
Total	53	82	135	

(Table. 4:3): EBV Immunostain, comparison between malignant lymphoma and the control group (normal and reactive lymph node)

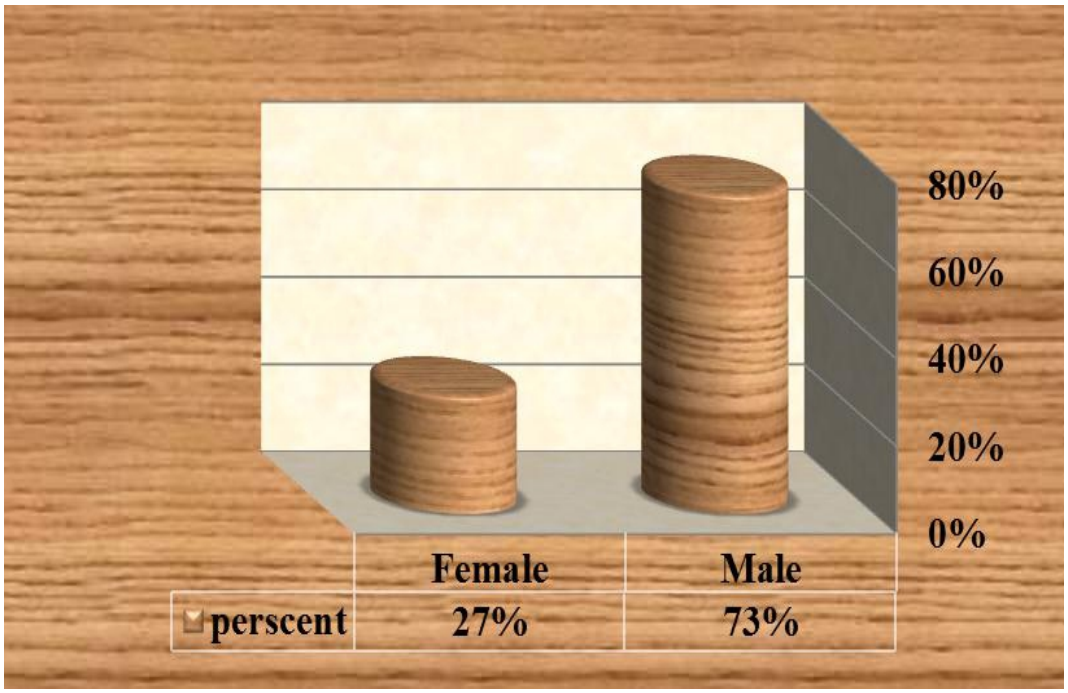
Study group	IHC (EBV) result		Total	p. value
	Positive	Negative		
Malignant lymphoma	23	47	70	**0.04**
reactive lymph note	2	18	20	
Total	25	65	90	

(Table.4: 4): EBV PCR, comparison between malignant lymphoma and the control group (normal reactive lymph node)

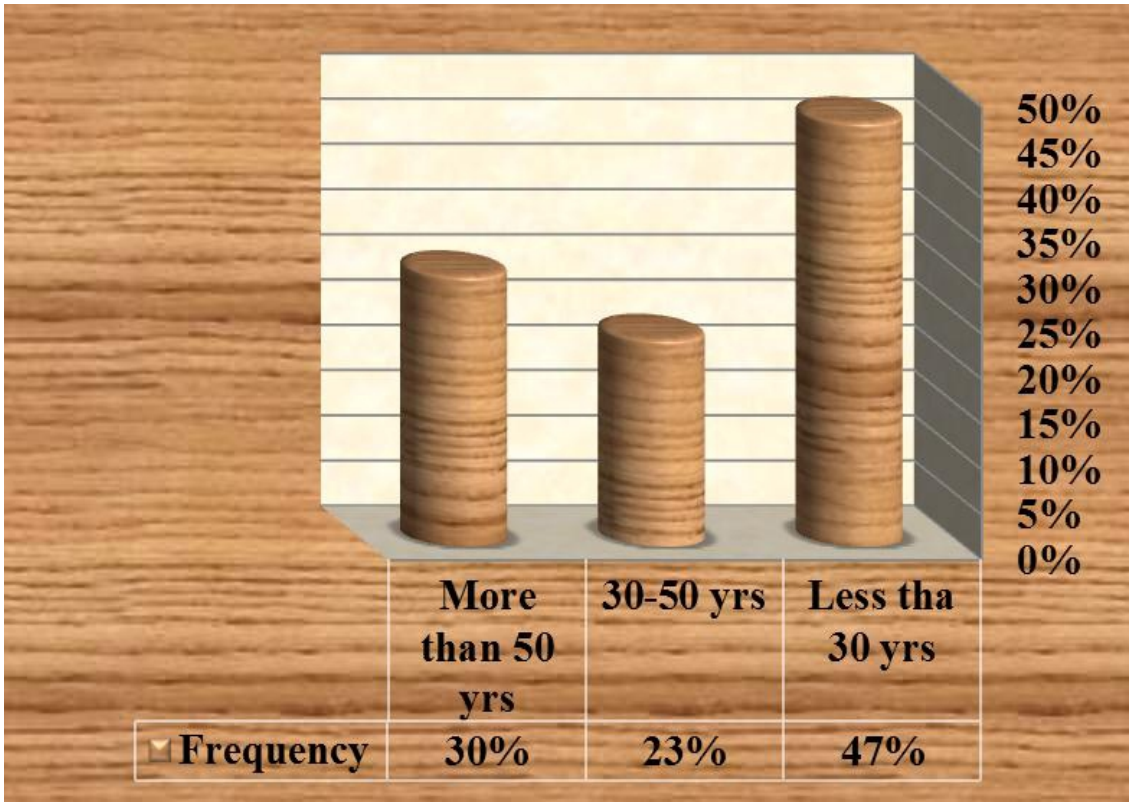
Study group	PCR (EBV) result		Total	p. value
	Positive	Negative		
Malignant lymphoma	29	41	70	**0.18**
reactive lymph note	5	15	20	
Total	34	56	90	



(Figure.4.3): the frequency of malignant lymphoma types



(Figure.4.4) the frequency of sex groups among malignant lymphomas



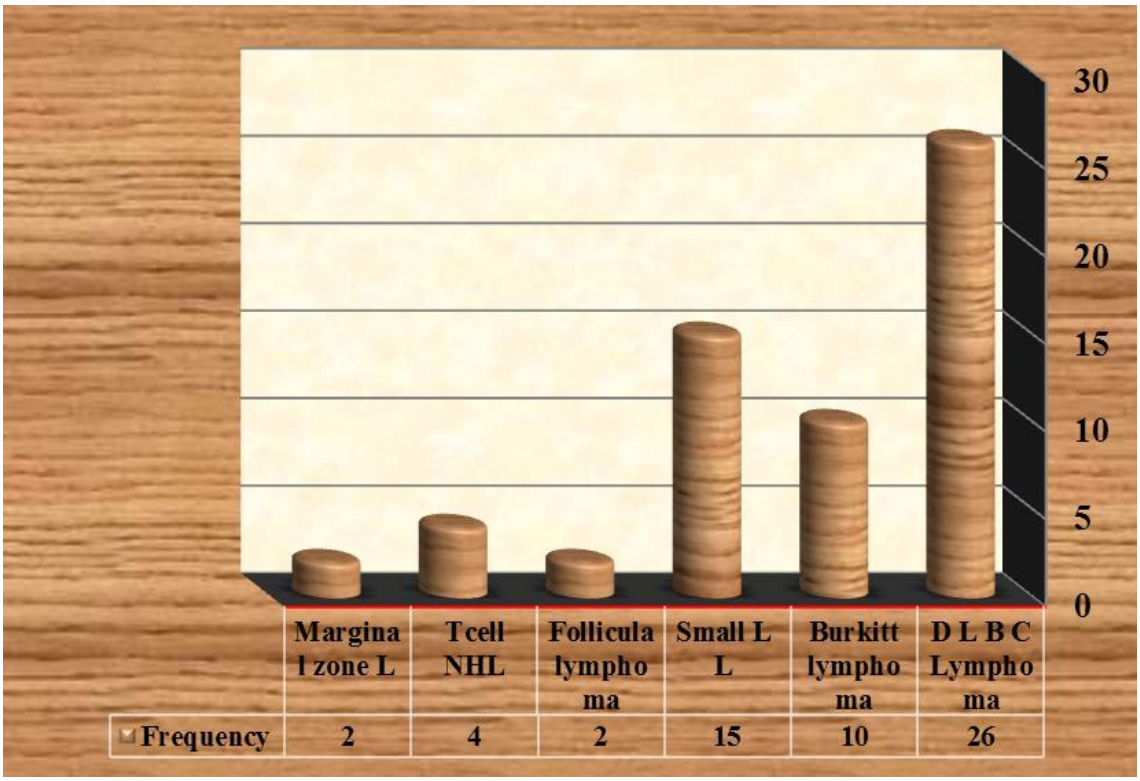
(Figure.4.5): the frequency of age groups among malignant lymphomas

(Table.4.5): EBV Immunostain , comparison between Hodgkin`s lymphomas and Non-Hodgkin`s lymphomas

Type of Lymphoma	IHC (EBV) result		Total	p. value
	Positive	Negative		
H.L	5	6	11	**0.333**
N.H.L	18	41	59	
Total	23	47	70	

(Table.4.6): EBV PCR , Comparison between Hodgkin`s lymphomas and N on Hodgkin`s lymphomas

Type of Lymphoma	PCR (EBV) result		Total	p. value
	Positive	Negative		
H.L	7	4	11	**0.103**
N.H.L	22	37	59	
Total	29	41	70	



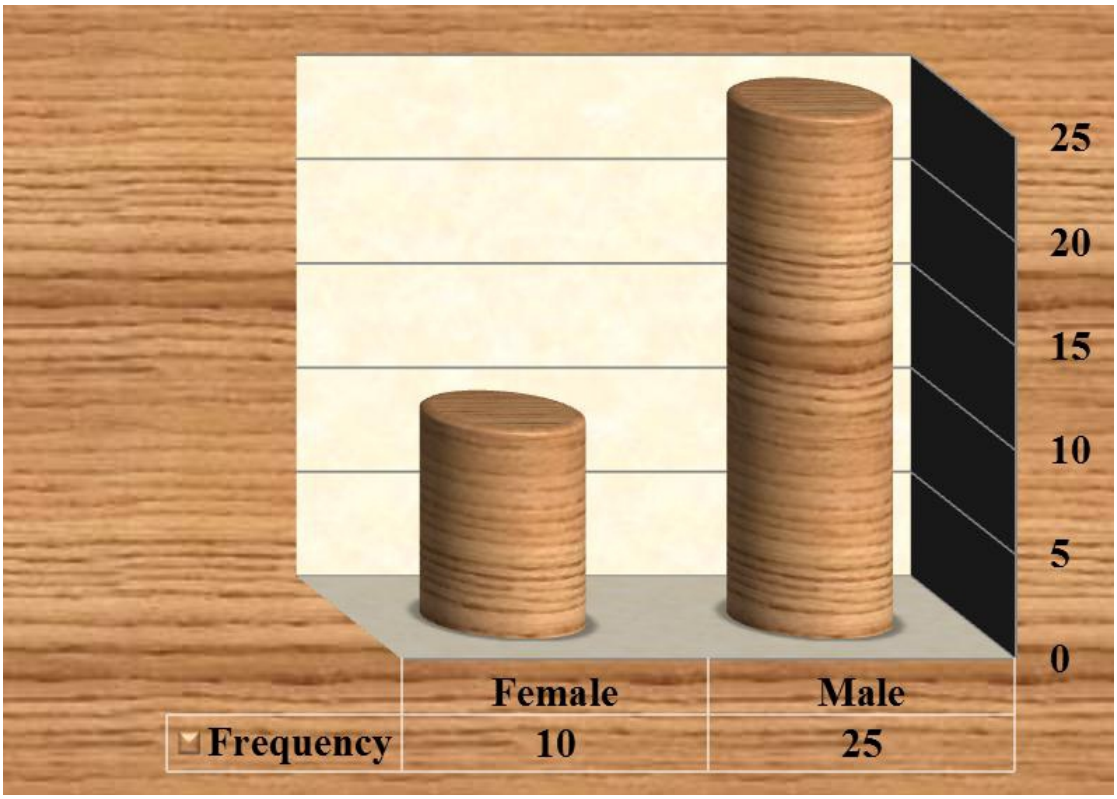
(Figure.4. 6): The frequency of Non-Hodgkin`s Lymphomas types

(Table .4.7): the association of age and Different types of N on Hodgkin`s lymphomas

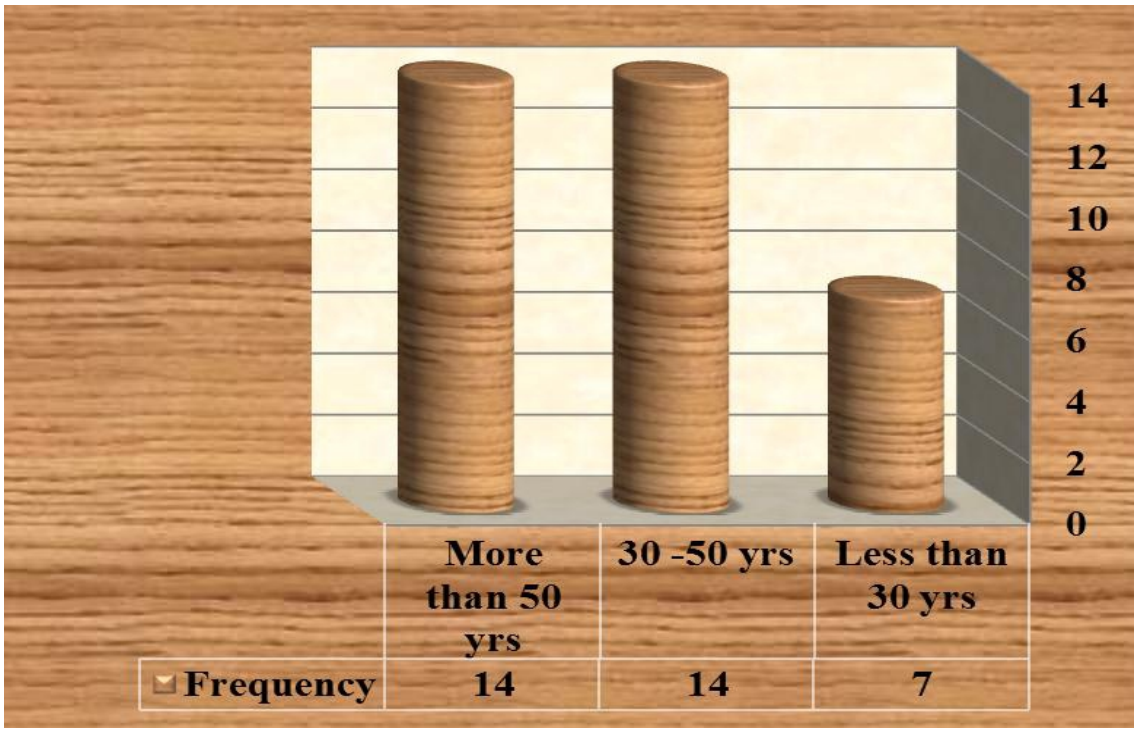
Type of NHL	Age group			Total	P.value
	Less than 30yrs	30 to 50 yrs	More than 50yrs		
D L B cell lymphoma	14	6	6	26	
Burkitt lymphoma	9	1	0	10	
Small .L lymphoma	1	3	11	15	
Follicular lymphoma	0	2	0	2	
T cell NHL	1	2	1	4	0.000
Marginal zone lymphoma	0	0	2	2	
Total	25	14	20	59	

(Table.4.8): the gender frequency , Comparison between Different types of Non Hodgkin`s lymphomas.

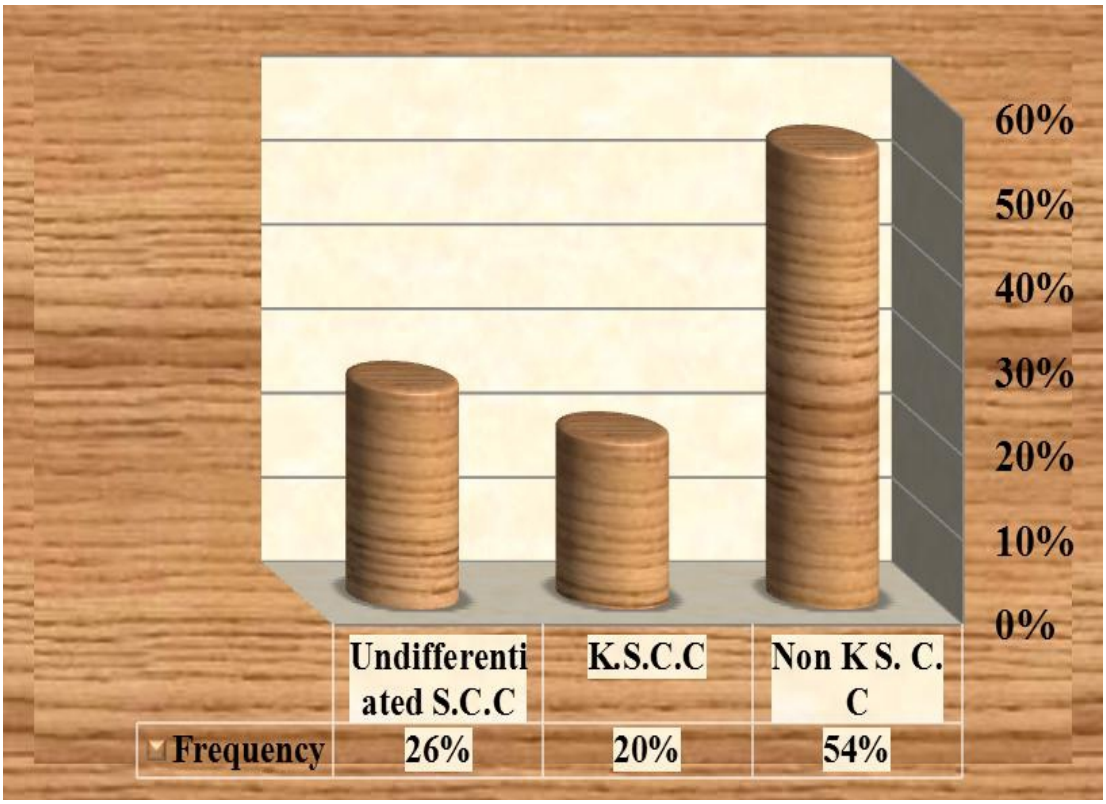
Type of NHL	Age group		Total	P.value
	Male	Female		
D L B cell lymphoma	16	10	26	0.5
Burkitt lymphoma	8	2	10	
Small .L lymphoma	10	5	15	
Follicular lymphoma	1	1	2	
T cell NHL	4	0	4	
Marginal zone lymphoma	2	0	2	
Total	41	18	59	



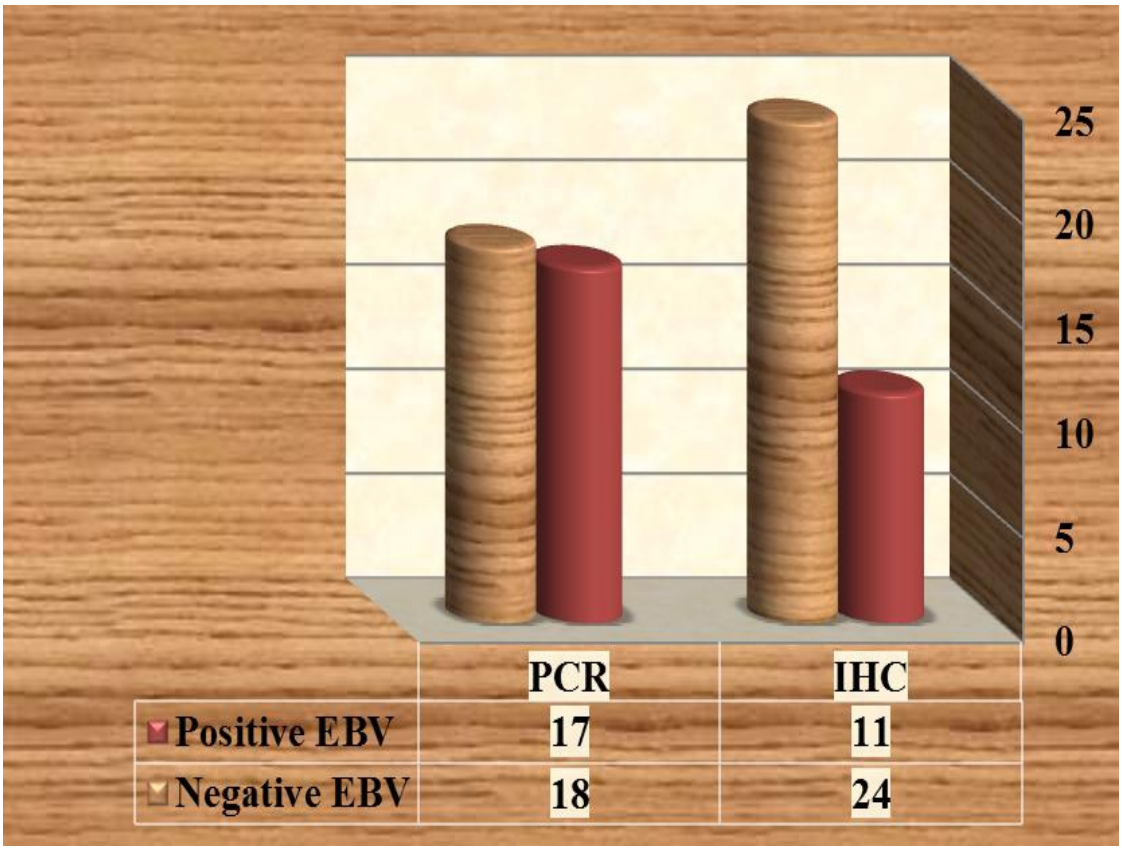
(Figure.4.7): Frequency of sex group among case of nasopharyngeal carcinoma



(Figure.4.8): Frequency of age group among case of nasopharyngeal carcinoma



(Figure .4.9): Frequency of histological types of nasopharyngeal carcinomas



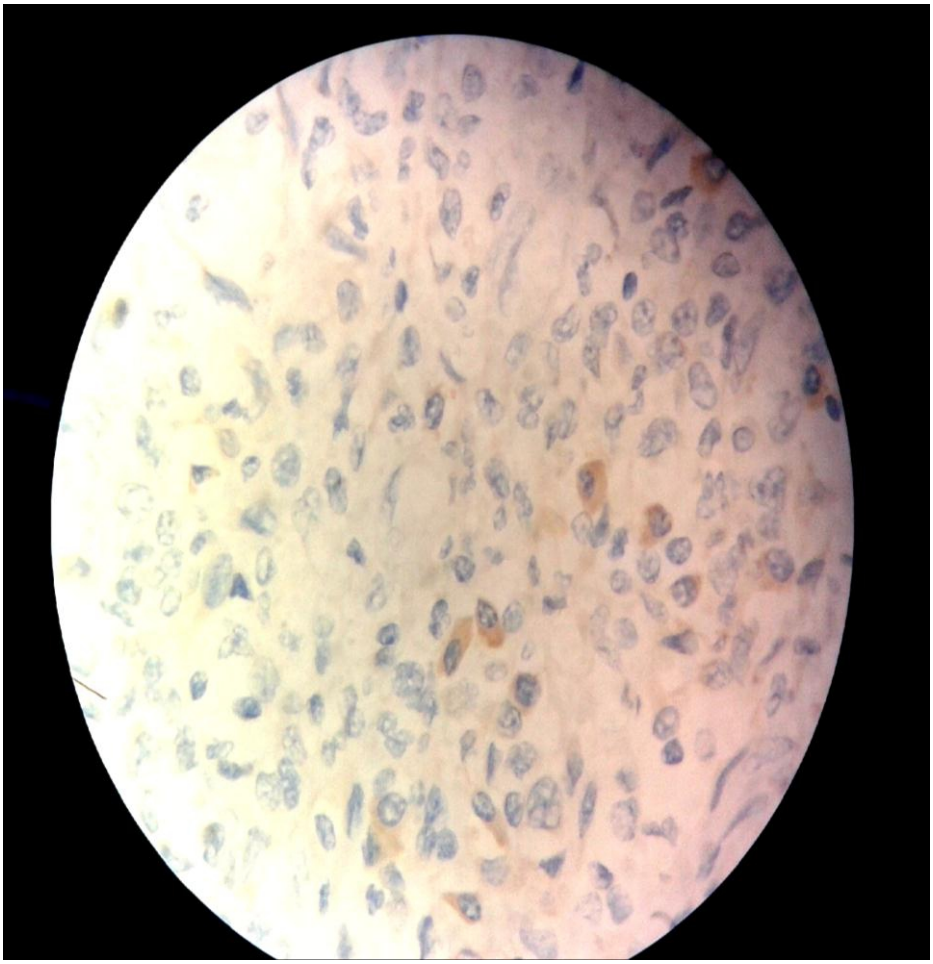
(Figure.4.10) Comparison between Immunostain and PCR EBV result among nasopharyngeal carcinomas

(Table.4.9): EBV IHC, comparison between different histological types of NPC

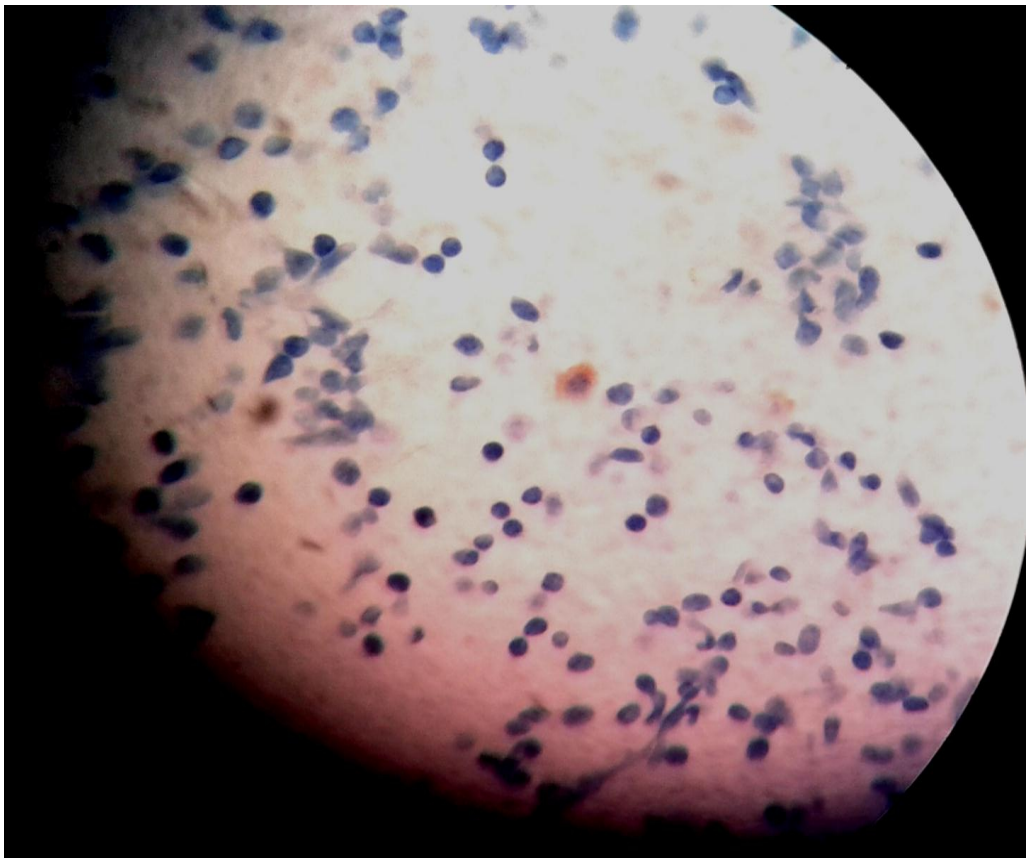
Diagnosis	IHC		Total	P.value
	+ve	-ve		
Non K S. C. C	4	15	19	0.182
K S. C. C	2	5	7	
Undifferentiated S.C.C	5	4	9	
Total	11	24	35	

(Table.4.10): EBV PCR, comparison between different histological types of NPC

Diagnosis	PCR		Total	P.value
	+ve	-ve		
Non K S. C. C	5	14	19	**0.001
K S. C. C	3	4	7	
Undifferentiated S.C.C	9	0	9	
Total	17	18	35	



Photograph .4.1: Immunostain positive reaction in Hodgkin lymphoma.



Photograph .4.2: Immunostain positive reaction in nasopharyngeal cancer

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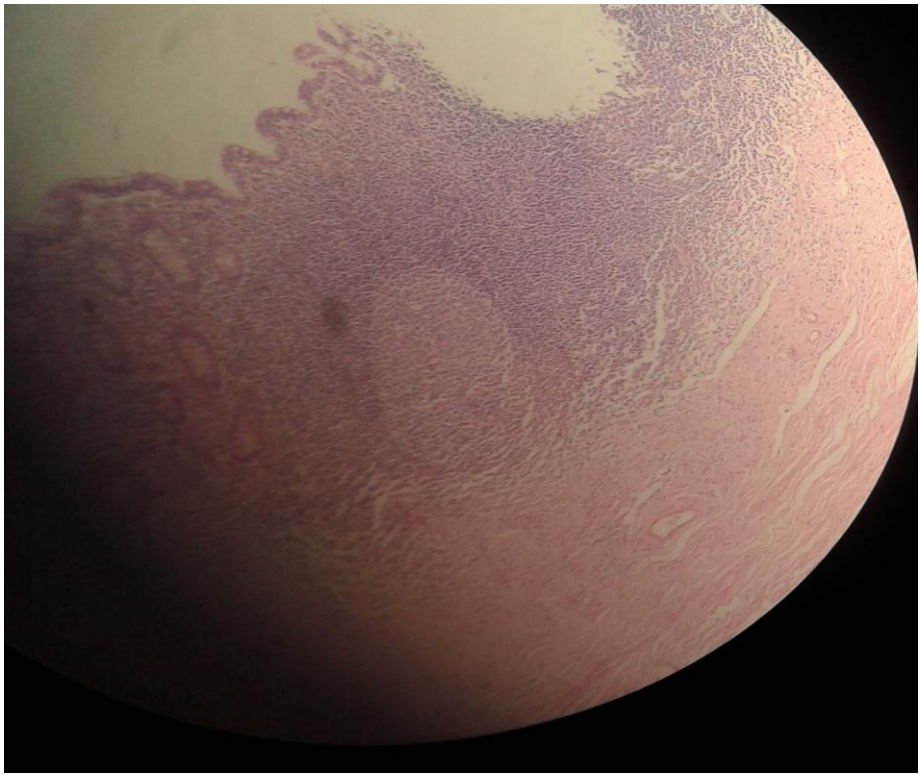
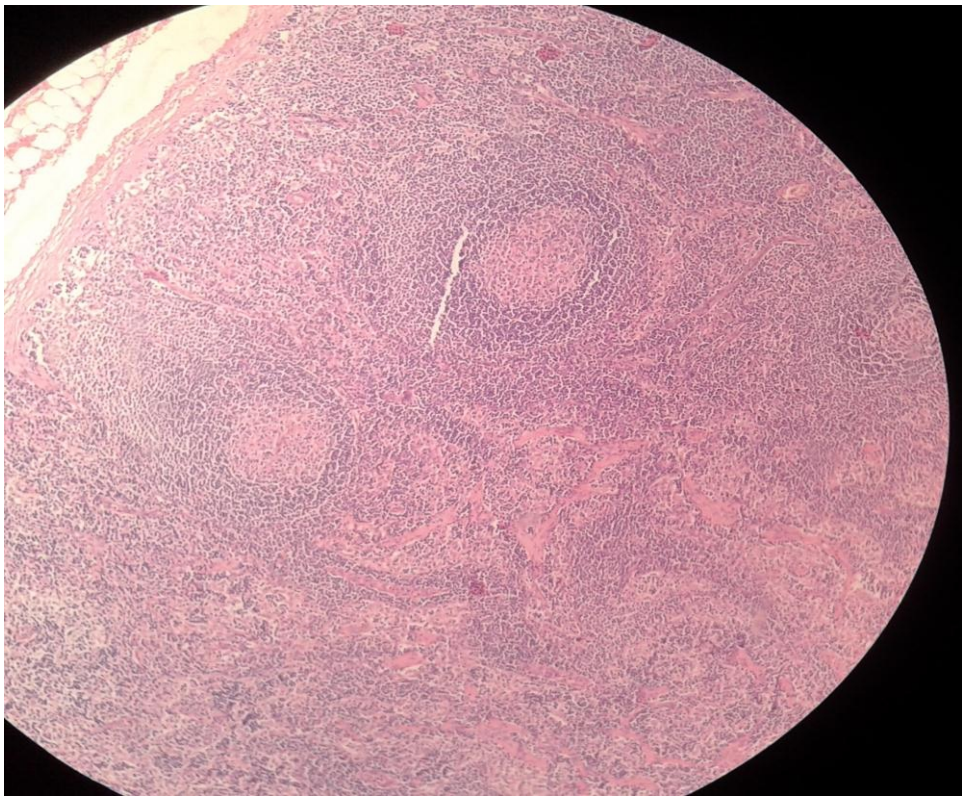
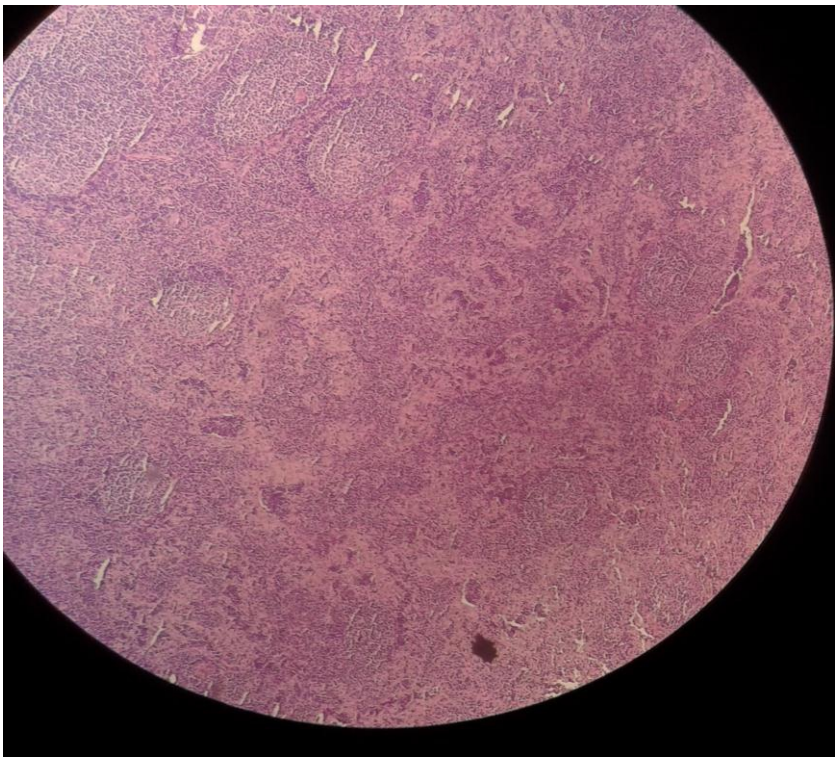


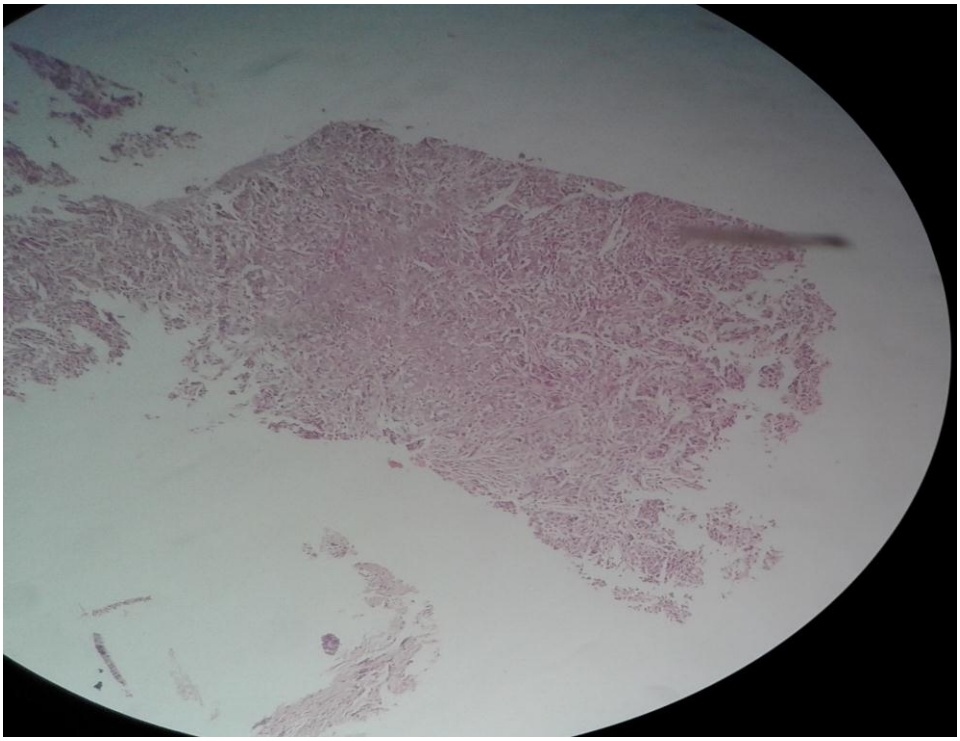
Photograph .4. 3: Normal lymphoid tissue from appendix, low power field magnification x10 (H&E)



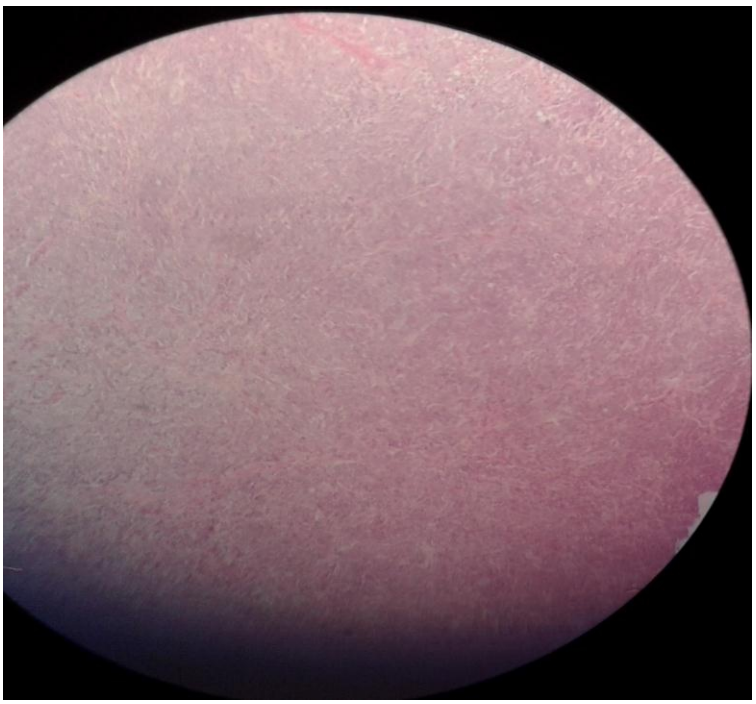
Photograph .4.4: Reactive lymphoid tissue, showed evenly distributed follicles with germinal center formations, low power field magnification x10 (H&E)



Photograph .4.5: Follicular lymphoma: lymph node effaced by small follicle .low power field magnification ,x10(H&E)

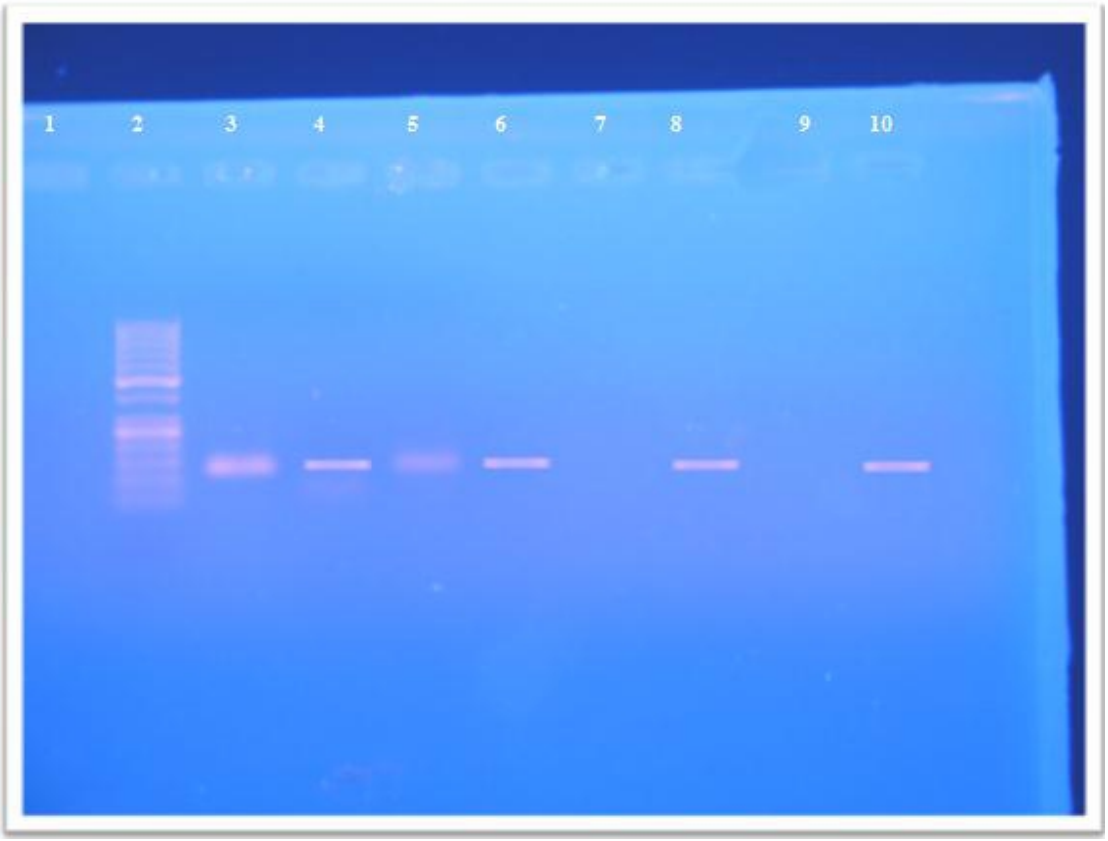


Photograph .4.6: (NPC). Undifferentiated squamous cell carcinoma: malignant pleomorphic cells with high N/C and hyperchromatic nuclei . low power field magnification.x10, (H&E)



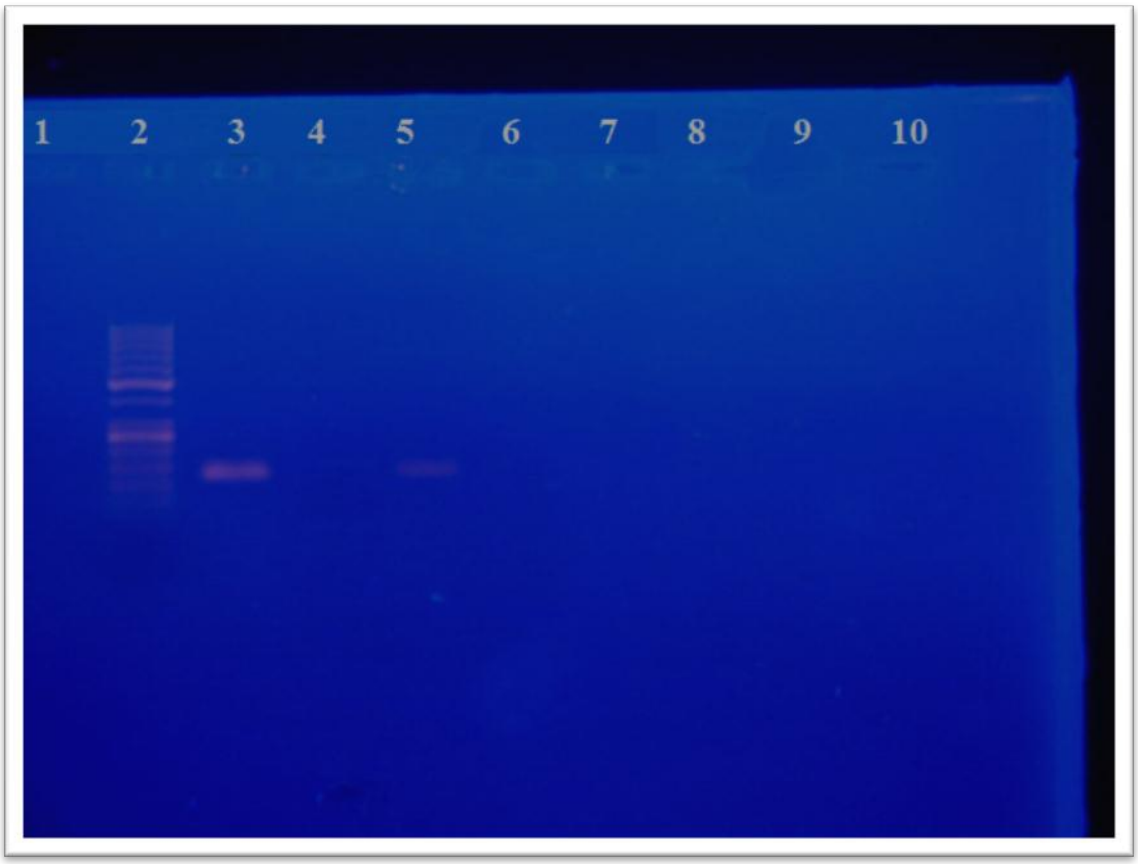
(Photo graph .4.7): Nasopharyngeal carcinoma, keratinized form: tissue composed of sheet of malignant squamous cells with scant keratin formations . low power field magnification. X10. (H&E)

PCR on gel documentary



1- Negative control
2- 100pb DNA marker
3- Positive control
4- , 5, 6, 8 and 10 Positive sample
7 and 9 Negative

PCR on gel documentary



1- Negative control
2- 100pb DNA marker
3- Positive control
4- Negative sample
5- Positive sample
6- To 10- negative sample

Discussion

This retrospective and prespective study was conducted in Khartoum state in the period from October 2010 to January 2015, the main aim of this study to investigate the association between EBV infection and lymphoma and nasopharyngeal carcinomas.

One hundred and thirty five (135), cases of formalin fixed paraffin embedded samples (*FFPE*), were investigated for EBV ,detected by LMP-1 immunohistochemistry and PCR targets the EBV gene encoding the viral latent membrane protein one (LMP-1). Clinical and pathological aspects such as age, sex and histological types were collected from clinical records of patient files. The collected data were representing information during four years from (2010 to 2014) , there were(52%) (70/135) of malignant lymphomas and (26%) (35/135) of nasopharyngeal carcinomas as (study groups), while (22%) (30/135) were non malignant samples include reactive lymph nodes hyperplasia and normal nasopharynx tissue, as (control groups), the study correlated the results with age, histological subtypes and gender , according to the demographic characteristic of the study group. In this study, males were predominates populations comprised (68%) and (32%) females, with ratio about 2:1.

The findings point out that, positive (LMP-1) Immunostain reaction for EBV, were reported in thirty four biopsies (32%) (34/105) among malignant tissues, compared with four positive (13%) (4/30) of non malignant tissue, indicating a significant statistical difference, the (P.value =0.04); While the positive PCR for EBV were reported in forty six (43.8%) (46/105) in malignant tissues, compared with seven (23.3%) (7/30) were positive in non malignant tissues , that means a significant difference between case and control groups in the association with EBV infection ,the (P.value= 0.043), so there is sufficient evidence for the carcinogenicity of EBV in the causation of lymphomas and nasopharyngeal carcinomas , this finding is supported by (Jackson Orem, *et al* . 2007) , their conclusion " Evidence for a causal relationship between EBV and

Burkitt lymphoma in the endemic form is fairly strong. Frequency of association between EBV and Burkitt lymphoma varies between different patient groups and different parts of the world. EBV may play a role in the pathogenesis of Burkitt lymphoma by deregulation of the oncogenic c-MYC by chromosomal translocation" .Also the strong evidence of EBV carcinogenicity came from (Gerald Niedobitek *et al*. 2001) they concluded "There is good evidence that EBV infection precedes expansion of the malignant cell populations in some virus-associated tumours. However, this is clearly not always the case and for some of these tumours there are indications that clonal genetic alterations may occur prior to EBV infection. Thus, whilst there is good evidence to suggest that EBV is a human carcinogen, its precise role in the development of virus-associated human tumours".

The data of this study provided evidence that PCR method yields a higher EBV-positive detection rate (43.8%) among study group compared with (32%) obtained by immunohistochemistry, indeed it is reported that the immunohistochemical technique may be less sensitive than PCR, this result supported by (Zong , *et al* ,2013) .Their study was conducted in China, and revealed that the PCR method presented the highest(74.6%) (44/59) detection rate compared to immunohistochemistry and In situ hybridization methods, that identified 66.1% (39/59, LMP1) and 67.8% (40/59, EBER1 ISH), EBV-positive results, respectively. The reason for this result may be that the target DNA is able to be amplified by thousands of times by the PCR procedure, thus the PCR method have a higher sensitivity than IHC. However, the PCR method was unable to provide definite information concerning the cellular localization of the EBV-positive cells as IHC do that, and at the same time the obtained results explained that LMP-1 Immunohistochemistry is an efficient tool to demonstrate presence of EBV.

Moreover the study showed that, the malignant lymphomas had higher frequency in male than female (73% versus 27%) the ratio: 2.7:1), similar findings were reported from Iran, by (Amir, *et al.* 2012), their results were "out of the 30 patients with primary GI lymphoma in the study, 12 were female (40%) and 18 were male (60%) (male to female ratio: 3/2). The reason for this gender domination in lymphoma may be due to increase exposure to the environmental factors, this explanation, supported by Canadian study by (Chandima, *et al*. 2008) whom concluded that, an increased risk of developing NHL is associated with the following: long held occupations of faer and machinist; exposure to diesel fumes; and exposure to ionizing radiation (radium). The risk of NHL increased with the duration of employment as a farmer or machinist.).

The study revealed that, the most commonly affected age group in lymphoma, was children and young adults, this finding similar with that reported in first National Population-based Cancer Registry (2009), in Sudan (in children less than 15 years of age, leukemia was the most common cancer followed lymphoma) .

The most predominant subtypes was Non Hodgkin lymphoma (84%) ,while Hodgkin lymphoma was (16%) .This finding was consistent with study done in China by (Wang, *et al*. 2006) as their results demonstrate that (86.1%) were confirmed to be non-Hodgkin lymphoma, while (13.9%) were Hodgkin lymphoma.

The present study clarified that LMP-1(EBV) immunohistochemistry , positive rate was (45%) for HL, and (31%) for non Hodgkin lymphoma , there was no significant different between HL and non HL existed as regard to association of EBV as the (P.value =0.333). The same finding but slightly higher was obtained by PCR , (63.6% and 37.3%) for HL and non HL respectively , these findings were in agreement with study conducted by (Córdova *et al*. 2003) , " they found LMP-1 positive rate of 50% for 22 cases of

HL and 35% for 63 cases of NH" .Other support results ,came from Hungary in study done by (Keresztes, *et al*. 2005) "with the finding that (56%) of HL cases, was EBV positive by PCR, while (43%) of HL cases was LMP-1 immunostain positive.

Globally, NHL is more common in males as compared to females. Similar trend was seen in this study in which (79.5%) of patients were males and (19.5%) were females. These results were similar to the findings of (Mushtaq, *et al*. 2008; Jamal *et al*. 2006), "in which males comprised 68% of the patients". In addition to that, the present study represent that , a diffuse Large B-cell lymphoma was the most common type of NHL (44%), followed by small lymphocytic lymphoma (25.4%), Burkitt lymphoma (17%), follicular lymphoma (3.3%), T-lymphoblastic lymphoma (7%), marginal zone lymphoma (3.3%) . These results were similar to the international data of Armitage *et al* (Armitage et al., 1997), "whose study also showed DLBCL (31%) to be most common type of NHL". ,but were inconsistent with frequency of Burkitt lymphoma (<1%). as the present study reveal higher frequency among Sudanese patients (17%),The same result was obtained in Pakistan by (Ishtiag, *et al*. 2013) "who concluded that,(the most common subtypes of non Hodgkin lymphoma is diffuse large B cell lymphoma". The explanation for variations in the prevalence of NHL in various studies might be due to different study methodologies of classifications, difference in sample size, and it also may be due to some environmental factors.

The study also investigates the nasopharyngeal carcinoma and its association with EBV infection. NPC forms around 6% of all cancer cases in the Sudan Cancer Registry (SCR) records, and (7%) at the Radiation Isotope Center Khartoum (RICK).Sudan is categorized as an intermediate risk country (Abuidris, *et al*. 2008).In this study, males were more affected than females (71% versus 29% respectively) with a sex ratio of 2.5. These findings were in agreement with Sudanese published study by (Ameera, *et al*. 2014), their results showed that; "in the gender frequency of patients with NPC, (79.1% males

versus 20.9% female). Such a male predominance in the incidence of NPC may be partly explained by differences between the sexes in the prevalence of some environmental risk factors, such as smoking and hazardous occupational exposures. It is also possible that some intrinsic exposures, such as sex hormones, could account for the observed male predominance by a protective effect of endogenous estrogen, this explanations supported by (Shao, *et al* . 2013) (The age-dependent pattern of the sex difference in the incidence of NPC could not be completely explained by known risk factors for NPC. The contributions of intrinsic exposures, such as sex hormones, merit consideration)

In this study most patients with NPC were in middle age and elderly, these findings were in line with study conducted in Indonesia by (Adham, et al. 2012) , "In this study population, NPC was the most frequent Head and neck cancer (28.4%), with a male-to-female ratio of 2.4, NPC appeared to affect patients at a relatively young age".

As considering the histological subtypes of NPC ,the study emphasized the predominance of non keratinized squamous cell carcinomas(54%) followed by undifferentiated squamous cell carcinoma (26%), then keratinized squamous cell carcinomas(20%) , this result resembles that of endemic areas, with a predominance of Types III and II, which associated with high rates of EBV detection (Chou, *et al*. 2008).

As regard to histological subtypes of NPC ,the PCR detected EBV DNA in (100% , 43% , 24%) of cases of undifferentiated squamous cell carcinoma , keratinized squamous cell carcinoma and in Non keratinized squamous cell carcinoma respectively , reflecting highly statistical significant ,with(P.value 0.001), while the LMP-1 immunostain reveal positive rate of EBV of (55%, 28% , 21%), without statistically signinificant differences with (P.value=0.182,). These findings agreement with the study conducted in Sudan by (Ameera, *et al*. 2014) "as the undifferentiated form record (100%) EBV positive , detected by (EBER-ISH) for EBV demonstration " .The study data

were also supported by findings of (Vera *et al* 1998), as their conclusions showed (76.36%) of NPC cases positive nuclear signals were observed using EBV-ISH. Overall, EBV-ISH positivity varied according to histological type, in that undifferentiated carcinomas showed higher proportion of positive cases than differentiated cell carcinomas".

Other study by (Hording, *et al*. 1993) confirmed the association of undifferentiated squamous cell carcinoma subtype of NPC and EBV infection (100%), but less frequent one with keratinizing NPC (14%) The results may indicate a different carcinogenesis for the WHO 1 NPC subtype.

Conclusions

- There is sufficient evidence for the carcinogenicity of EBV in the causation of lymphomas and nasopharyngeal carcinomas**,** as demonstrated by LMP-1 Immunostain and EBV (DNA) PCR.
- PCR targets the EBV gene encoding the viral latent membrane protein one (LMP-1) is more sensitive than LMP-1 Immunostain.
- The malignant lymphomas had higher frequency in males than female. The commonly affected age was children and younger patients, and the most predominant subtype was non Hodgkin lymphoma (84%).
- Malignant lymphoma was highly associated with EBV infection regardless of histological subtypes.
- NHL is more common in males as compared to females, and diffuse Large B-cell lymphoma was the most common sub type
- The nasopharyngeal carcinomas, commonly affect males. The most commonly affected age groups were middle age and elders, and the predominance subtype was non keratinized Squamous Cell Carcinomas. Higher EBV positive rate was detected in undifferentiated squamous cell carcinoma subtype.

Recommendations

- Adopting EBV screening program, among high risk populations, especially children.
- Further studies should be done to find out whether this positivity is due to co-infection or have a role in pathogenesis.
- Further studies should be done to investigate EBV, using other techniques, of the molecular especially (Insitu hybridization technique) and biological changes, that occurs during carcinogenesis and progression of cancers.
- LMP-1 immunostain should be apply as a marker of EBV infection

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بسم الله الرحمن الرحيم

University of Shendi

Post graduate college

Faculty of medical laboratory sciences

Immunohistochemical Detection of Epstein - Barr virus (EBV) Among Sudanese Patients with Lymphoma and Nasopharyngeal Carcinoma

1. **Demographic Data:**

Code: …….

1- Patient name----------

2- Patient age ----------

3- Gender: Male ( ) Female ( )

4- Lab No----------

5- Phone NO----------

6- Address----------

7- Tribe ----------

8- **Occupation ----------**

2. **Analytic Data:**

9- In case of Lymphoma

a- Site of tissue taken biopsy

1-cevical lymph node ( ) 2-axillary lymph node( ) 3-inguinal lymph node( )

4-Tonsil lymph node ( ) 5-others----------

b-What type of Lymphoma: HL ……..

NHL B cells …….

NHL T cells…….

Other ……....

c- What is the performed tumor marker----------?

d- Tumor grade ----------

In case of Nasopharyngeal Carcinoma

I. **What is affected organ ----------**

II. **What type of Cancer**:

a. Keratinizing squamous cell carcinoma (KSCC, formerly WHO type I ( )

b. Non-keratinizing carcinoma differentiated type (NK-D, formerly WHO type II ( )

c. Non-keratinizing carcinoma undifferentiated (NK-U, formerly WHO type III) ( )

III. **What is the performed tumor marker----------**

IV. **Tumor grade----------**

V. **Others----------**

Solution preparations

Preparation of Mayer`s Hematoxylin

Hematoxylin	1 g
Distilled water	1000 ml
Potassium or ammonium alum	50 g
Sodium iodate	0.2 g
Citric acid	1 g
Chloral hydrate SLR	50 g

Immunochistochemical method for nasopharyngeal carcinoma and the controls:

Applications:

Anti-Epstein-Barr virus antibody targets the 60 kDa latent membrane protein (LMP-1) encoded by the BNLF1 gene of the Epstein-Barr virus.

Presentation:

Anti-Epstein Barr Virus is a cocktail of four mouse monoclonal antibodies from tissue culture supernatant diluted in tris buffered saline, pH 7.3-7.7, with protein base, and preserved with sodium azide.

Reactivity: Paraffin, frozen

Control; Hodgkin's disease, infected tissue

Visualization; Cytoplasmic

Stability Up to 36 months; store at 2-8°C

Isotype IgG_1

Description Ventana®* Cat. No.760-2640

Recommended Ventana®* auto stainer machine was applied (Index) Staining Procedure (Ultra View™)

1. Load slides, antibody, and Ultra View™ detection kit dispensers onto Bench Mark®* instrument.
2. Select CC1 Standard pretreatment.
3. Antibody incubation should be set for 32 minutes at 37° C.

4. Start the run.
5. When the staining run is complete, move slides from instrument and rinse well with wash buffer.
6. Cover slip

DNA Extraction:

DNA was extracted according to the steps described in DNA extraction kit that purchased from Bio Basic inc. One –Tube DNA Extraction Kit (Version 5.0).

Introduction about One –Tube DNA Extraction Kit:

The kit is designed for rapid isolation of genomic DNA from animal tissue. There is no need for phenol extraction, overnight digestion, DNA precipitation or column purification; the lysate can be use directly as PCR template

Kit contents

Components	BS8401/100 Preps
Lysis- Buffer –T	20 ml
Proteinase K	2 ml
Universal Buffer NST	20 ml

PROCEDURE

STAINING PROTOCOL (kit components in bold):

1. Deparaffinize and rehydrate tissue section.
2. To reduce nonspecific background staining due to endogenous peroxidase, incubate slide in hydrogen peroxide for 10-15 minutes.
3. Wash 2 times in buffer.
4. If required, incubate tissue in digestive enzyme (or appropriate pretreatment).
5. Wash 4 times in buffer.
6. (Optional) Apply **Ultra V Block** and incubate for 5 minutes at room temperature to block nonspecific background staining. **NOTE:** Do not exceed 10 minutes or there may be a reduction in desired stain.
7. Rinse (Optional).
8. Apply primary antibody and incubate according to manufacturer's protocol. 30
9. Wash 4 times in buffer.
10. Apply **Biotinylated Goat Anti-Polyvalent** and incubate for 10 minutes at room temperature.
11. Wash 4 times in buffer.
12. Apply **Streptavidin Peroxidase** and incubate for 10 minutes at room temperature.
13. Rinse 4 times in buffer.
14. Incubate with peroxidase-compatible chromogen of choice according to manufacturer's recommendations.
15. Counterstain and coverslip.

The specificity and sensitivity of antigen detection is dependent on the specific primary antibody used.

REFERENCES

N/A

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INTENDED USE

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AVAILABILITY:

Catalog #	Slide Volume
TP-060-HL	300-600 slides
TP-125-HL	625-1250 slides

SPECIFICITY: Anti-Mouse IgG (H+L), Anti-Rabbit IgG (H+L)
ENZYME: Peroxidase
CHROMOGEN/SUBSTRATE: None provided

REAGENTS

Qty.	Component	TP-060-HL	TP-125-HL
1	Ultra V Block	TA-060-UB	TA-125-UB
1	Biotinylated Goat Anti-Polyvalent	TP-060-BN	TP-125-BN
1	Streptavidin Peroxidase	TS-060-HR	TS-125-HR

(The three-digit number in the middle of each Catalog # designates the reagent volume in mL or number of tablets.)

DESCRIPTION

The reagents in this kit constitute a labeled streptavidin-biotin immunoenzymatic antigen detection system. This technique involves the sequential incubation of the specimen with an unconjugated primary antibody specific to the target antigen, a biotinylated secondary antibody that reacts with the primary antibody, enzyme-labeled streptavidin, and substrate-chromogen.

PRINCIPLE OF THE PROCEDURE

This UltraVision detection system detects a specific antibody bound to an antigen in tissue sections. The specific antibody is located by a biotin-conjugated secondary antibody. This step is followed by the addition of a streptavidin-enzyme conjugate that binds to the biotin present on the secondary antibody. The specific antibody, secondary antibody, and streptavidin-enzyme complex is then visualized with an appropriate substrate/chromogen.

WARNINGS & PRECAUTIONS

Refer to MSDS.

STORAGE & SHELF LIFE

Store at 2-8°C. Each component is stable for 18 months.

MICROBIOLOGICAL STATE

Product(s) not sterile.

MATERIALS REQUIRED BUT NOT PROVIDED

Primary antibody. Diluent.

SPECIMEN & REAGENT PREPARATION

Refer to Procedure.

Lab Vision Corporation
46360 Fremont Blvd.
Fremont, CA 94538-6406, USA
US Toll Free: 1 (800) 522-7270
Phone: +1 (269) 544-5600
Fax: 1 (269) 372-2674
www.thermoscientific.com/labvision

EC REP

Thermo Fisher Scientific
Anatomical Pathology
Tudor Road, Manor Park
Runcorn, Cheshire WA7 1TA, UK
Tel: +44 (0) 1928 534 050
Fax: +44 (0) 1928 534 049
sales.ap.uk@thermofisher.com

Thermo SCIENTIFIC

IN VITRO DIAGNOSTIC DATA SHEET
Rev 032312C

Epstein-Barr Virus / LMP Ab-1

Catalog # MS-1458-S0, -S1, or -S (0.1ml, 0.5ml, or 1.0ml)
Catalog # MS-1458-R7 (7.0ml)

Please note this data sheet has been changed effective March 23, 2012.

INTENDED USE:

- ***For In Vitro Diagnostic Use:*** This product is intended for qualitative immunohistochemistry with normal and neoplastic formalin-fixed, paraffin-embedded tissue sections, to be viewed by light microscopy.

Description: Ab-1 reacts strongly with EBV-positive lymphoblastoid cell lines and EBV infected B cell immunoblasts in infectious mononucleosis. It also reacts with 25 to 50 per cent of EBV-associated undifferentiated nasopharyngeal carcinomas and with Reed Sternberg cells in approximately 90% of EBV-associated Hodgkin's disease cases. The cocktail recognizes distinct epitopes on the hydrophilic carboxyl region of LMP which is exposed to the cytosol. On occasion, aberrant binding to brain and melanoma tissues can be seen.

- ***Expected Staining Pattern:*** Cytoplasmic
- ***Positive Control:*** EBV-positive lymphoma.

MATERIALS PROVIDED:

Epstein-Barr Virus / LMP Ab-1 (refer to catalog number):

- #MS-1458-S (or -S0, -S1): Tissue culture supernatant, concentrated, with 0.09% Sodium Azide.
  or
- #MS-1458-R7: (7.0ml) of antibody prediluted in 0.05mol/L Tris-HCl, pH 7.6 containing stabilizing protein and 0.015mol/L sodium azide.

- ***Antibody Concentration:*** Not known
- ***Host:*** Mouse
- ***Mol. Wt. of Antigen:*** 60kDa (LMP)
- ***Epitope:*** Located in the internal part of the membrane-associated viral LMP
- ***Species Reactivity:*** EBV-positive cells.
- ***Clone Designation:*** CS1+CS2+CS3+CS4
- ***Ig Isotype / Light Chain:*** IgG1 / kappa
- ***Immunogen:*** EBV-encoded recombinant latent membrane protein.
- ***Microbiological State:*** This product is not sterile.

MATERIALS REQUIRED, BUT NOT PROVIDED:

- ***Antibody Diluent:*** For concentrated antibodies, the antibody must be diluted before using. Use Lab Vision Antibody Diluent (catalog # TA-125-UD). Refer to diluent product instructions for use.
- ***Negative Control Reagent:*** Refer to the "General Protocol" instructions.
- ***Visualization System:*** Refer to the "General Protocol" instructions.

METHODS AND PROCEDURES:

Specimen Preparation	Refer to the "General Protocol" instructions.
Dilution of Concentrated Antibody	1:25-1:50 in antibody diluent
Tissue Section Pretreatment	Staining of formalin-fixed tissue sections requires treating the tissue sections in boiling 10mM citrate buffer, pH 6.0 (Lab Vision catalog # AP-9003), for 10-20 minutes followed by cooling at room temperature for 20 min.
Primary Antibody Incubation Time	60 minutes at Room Temperature
Visualization	To detect antibody, follow the instructions provided with the visualization system.

STORAGE and STABILITY:

This product contains sodium azide and is stable for 24 months when stored at 2-8°C. Do not use after expiration date indicated on label of the product. If reagent is not stored as recommended, performance must be validated by the user.

REFERENCES:

1) Rowe M et al. J Gen Virol 1987; 68:1575-86.

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Fax: +1 (269) 372-2674
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Thermo SCIENTIFIC

IN VITRO DIAGNOSTIC DATA SHEET
Rev 032312C

Epstein-Barr Virus / LMP Ab-1

Catalog # MS-1458-S0, -S1, or -S (0.1ml, 0.5ml, or 1.0ml)
Catalog # MS-1458-R7 (7.0ml)

Please note this data sheet has been changed effective March 23, 2012.

2) Liebowitz D et al. J Virol 1986; 58:233-7.

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can be use directly as PCR template. The one-tube procedure minimizes possibility of cross-contaminations between samples. The whole procedure takes less than 15 minute and this kit is an example of speed and efficiency. The kit is suitable for high throughput PCR screening of large scale samples. This kit also works for other application such as Genotyping, Transgene screening, Knockout analysis and Sequencing.

Recommended Samples Size per Prep

- 0.3-0.5 cm mouse tail
- 0.5-2 mm mouse ear punch
- 2-5 mg piece of tissue
- 1-10 hairs with roots
- 20 µl saliva
- 2-3 mm^2 piece of zebrafish fin

Features

- ✓ Simple and rapid. Whole procedure can be performed in one tube, takes approx 15 minutes.
- ✓ The whole procedure is performed in one single tube to prevent cross-contamination among samples.
- ✓ Convenient for high-throughout PCR screening.
- ✓ Suitable for extraction of genomic DNA from various species.

Protocols for Tissue Sample

1. Add 100 µl Lysis-Buffer-T, 10 µl of Proteinase K solution to a 0.5 ml centrifuge tube. Mix by vortexing.

Note: For batch extractions, Lysis-Buffer-T and Proteinase K may be pre-mixed at a ratio of 10:1 prior to use.

2. Add sample into the buffer, ensure that the sample is fully submerged in the solution.

3. Incubate the sample at room temperature for 10 min.

Note: Incubation at 56°C may enhance tissue lysis and amplification.

4. Incubate the sample at 95°C for 3 min.

Note: Tissues may not be completely digested at the end of the incubation, but this does not affect PCR performance.

5. Add 100 µl Universal Buffer NST. Mix by inverting the tube for about 10 times or vortex briefly.

6. The mixture can be used as PCR template directly. The volume of this template should not exceed 1/10 of the total PCR reaction volume.

Note: No spin step is required.

7. Save the remaining samples at 4°C.

Note 1: The DNA is not sufficient for electrophoresis analysis.

Note 2: For long term storage, remove undigested tissue or transfer the extracts to new tubes. Store DNA at -20°C

Protocols for Buccal Swab

1. Collect buccal cells on swab and dry the swab at room temperature for about 10 minutes.

Note: A foam-tipped swab is recommended.

2. Add 200 µl Lysis-Buffer-T, 10 µl of Proteinase K solution to a 0.5 ml centrifuge tube. Mix by vortexing.

Note: For batch extractions, Lysis-Buffer-T and Proteinase K may be pre-mixed at a ratio of 10:1 prior to use.

3. Place the dried buccal swab into the prepared lysis solution for 2 minutes and rotate the swab in the solution at least 5 times.

4. Rotate and press the swab firmly against the side of the tube to ensure that most of the liquid remains in the tube. Discard the swab.

5. Incubate the sample at room temperature for 5 min.

Note: Incubation at 56°C may enhance tissue lysis and amplification.

6. Incubate the sample at 95°C for 3 min.

Note: Tissues may not be completely digested at the end of the incubation, but this does not affect PCR performance.

7. Add 200 µl Universal Buffer NST. Mix by inverting the tube for about 10 times or vortex briefly.

8. The mixture can be used as PCR template directly. The volume of this template should not exceed 1/10 of the total PCR reaction volume.

Note: No spin step is required.

9. Save the remaining samples at 4°C.

Note 1: The DNA is not sufficient for electrophoresis analysis.

Note 2: For long term storage, remove undigested tissue or transfer the extracts to new tubes. Store DNA at -20°C

Maxime PCR PreMix Series *Research Use Only*

ISO 9001/14001 Certified Company

Maxime PCR PreMix Kit (*i*-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) **Cat. No. 25026**(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
Maxime PCR PreMix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

CONTENTS

- *Maxime* PCR PreMix (*i*-Taq, for 20μl rxn) 96 (480) tubes
- *Maxime* PCR PreMix (*i*-Taq, for 50μl rxn) 96 tubes

Component in	20 μl reaction	50 μl reaction
i-Taq™ DNA Polymerase(5U/μl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   Note 1 : Recommended volume of template and primer : 3μl~9μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl . Do not calculate the dried components

Example **Total 20μl or 50μl reaction volume**

PCR reaction mixture	Add	Add
Template DNA	1 ~ 2μl	2 ~ 4μl
Primer (F : 10pmol/μl)	1μl	2 ~ 2.5μl
Primer (R : 10pmol/μl)	1μl	2 ~ 2.5μl
Distilled Water	16 ~ 17μl	44 ~ 41μl
Total reaction volume	**20 μl**	**50 μl**

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94℃	2min	2min	2min
30-40 Cycles	Denaturation	94℃	20sec	20sec	20sec
	Annealing	50-65℃	10sec	10sec	20sec
	Extension	65-72℃	20-30sec	40-50sec	1min/Kb
Final extension		72 ℃	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

- ***Comparison with different company kit***

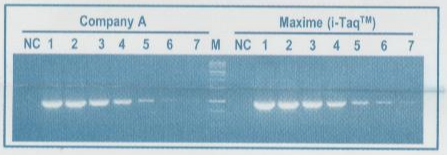

Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment .
After diluting the λDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product.
Lane M, SiZer-1000 DNA Marker; **lane 1,** undiluted λDNA; **lane 2,** 200 ng λDNA; **lane 3,** 40 ng λDNA; **lane 4,** 8 ng λDNA; **lane 5,** 1.6 ng λDNA; ; **lane 6,** 320 pg λDNA; **lane 7,** 64 pg λDNA; **lane NC**, Negative control

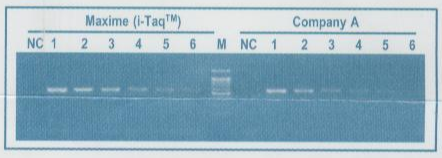

Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
lane M, SiZer-100 DNA Marker; **lane 1,** undiluted cDNA; **lane 2,** 1/2 diluted cDNA; **lane 3,** 1/4 diluted cDNA; **lane 4,** 1/8 diluted cDNA; **lane 5,** 1/16 diluted cDNA; **lane 6,** 1/32 diluted cDNA; **lane NC**, Negative control

iNtRON BIOTECHNOLOGY
www.intronbio.com / info@intronbio.com
T. (0505)550-5600 / F. (0505)550-5660

Code	Product Name	Size
BS88203	AIII-In-One DNA/RNA Mini-preps Kit	50 Preps
BS410A	EZ-RNA Reagents	100 ml
BS583	EZ-10 Spin Column Bacterial Total RNA Mini-Preps Super Kit	50 Preps
BS91915	EZ-10 Spin Column Fungal RNA Mini-Preps Kit	50 Preps
BS82314	EZ-10 Spin Column Plant RNA Mini-Preps Kit	50 Preps
BS82312	EZ-10 Spin Column Animal Total RNA Extraction Kit	50 Preps
BS82313	EZ-10 Spin Column Blood RNA Mini-Preps Kit	50 Preps
BS8856	EZ-10 Total RNA Mini-Preps Kit	50 Preps
BS88254	RNase-Free DNase Set	50 Preps
BS88133	EZ-10 Plus RNA Mini-Preps Kit	50 Preps
BS88136	EZ-10 Plus RNA Mini-Preps Kit	250 Preps

PRODUCTS ARE INTENDED FOR BASIC SCIENTIFIC RESEARCH ONLY!
NOT INTENDED FOR HUMAN OR ANIMAL USE!

Please visit www.biobasic.com

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BIO BASIC INC.

One-Tube Tissue DNA Extraction Kit

BS8401 and BS8402

Version 5.0
ISO9001 Certified

20 Konrad Cres, Markham Ontario L3R 8T4 Canada
Tel: (905) 474 4493, (800) 313 7224 Fax: (905) 474 5794
Email: order@biobasic.com Web: www.biobasic.com

One-Tube Tissue DNA Extraction Kit

Product information for BS8401/BS8402:

Kit Contents

Components	BS8401 100 Preps	BS8402 500 Preps
Lysis-Buffer-T	20 ml	100 ml
Proteinase K	2 ml	10 ml
Universal Buffer NST	20 ml	100 ml
Protocol	1	1

Storage and Stability

Transportation at ambient temperature. Store at 4°C, Valid for 1 year. . Proteinase K solution can be stored at 4°C for 6 months, or -20°C for long-term.

Introduction

The kit is designed for rapid isolation of genomic DNA from animal tissue, mouse tail and ear clips, human hair or saliva, fish and insect tissues. There is no need for phenol extraction, overnight digestion, DNA precipitation or column purification; the lysate

CELL MARQUE 6600 Sierra College Blvd. • Rocklin, CA 95677 USA • 800-665-7284

Rev. 2.1
KEY-CODE CMC26400021

Epstein Barr Virus (CS1-4)

For In Vitro Diagnostic Use (IVD)
English: Instructions For Use

Presentation
Anti-Epstein Barr Virus is a cocktail of four mouse monoclonal antibodies from tissue culture supernatant diluted in tris buffered saline, pH 7.3-7.7, with protein base, and preserved with sodium azide.

Applications
Anti-Epstein-Barr virus antibody targets the 60 kDa latent membrane protein (LMP-1) encoded by the BNLF1 gene of the Epstein-Barr virus. There is cross-reactivity with Reed Sternberg cells of Hodgkin's disease. The Epstein-Barr virus is an important as a cause of Infectious mononucleosis and has been associated with oral carcinomas.

Reactivity	Paraffin, frozen
Control	Hodgkin's Disease, infected tissue
Visualization	Cytoplasmic
Stability	Up to 36 months; store at 2-8°C
Isotype	IgG_1

Antibody color does not affect performance

Description	**Ventana®* Cat. No.**
50 test dispenser	760-2640

Preparation
1. Cut 3-4 µm section of formalin-fixed, paraffin-embedded tissue and place on positively charged slides; dry overnight at 58° C.

Recommended Ventana®* Staining Procedure (UltraView™)
1. Load slides, antibody, and UltraView™ detection kit dispensers onto BenchMark®* instrument.
2. Select CC1 Standard pretreatment.
3. Antibody incubation should be set for 32 minutes at 37° C.
4. Start the run.
5. When the staining run is complete, move slides from instrument and rinse well with wash buffer.
6. Coverslip.

Recommended Ventana®* Staining Procedure (iView™)
1. Load slides, antibody, and iView™ detection kit dispensers onto BenchMark®* instrument.
2. Select CC1 Standard pretreatment.
3. Antibody incubation should be set for 32 minutes at 37° C.
4. Start the run.
5. When the staining run is complete, move slides from instrument and rinse well with wash buffer.
6. Coverslip.

References
1. Murray PG et al. J Pathol. 166: 1-5 (1992)
2. Jarrett RF et al. Blood 78:1-10 (1991)
3. Pailesen G et al. Lancet. 337: 320-322 (1991)
4. Silverberg GS et al Principles and Practice of Surgical Pathology and Cytopathology, 3rd edition. (1997)

EC REP EMERGO EUROPE
Molenstraat 15, 2513 BH, The Hague, NL.

CE *Material Safety Data Sheet available upon request.*

Inline Dispenser Preparation, Handling & Storage Instructions

Preparing For Use:

Where Used: For NexES® IHC, BenchMark® Series and Discovery® automated instruments, software version 8.0 and higher.

STEP 1: Shipping Key Removal
To remove the Shipping Key (shown in Figure A), remove the Nozzle Cap, hold the dispenser upright and pull the Key Tab to disengage it from each end. DO NOT cover the nozzle tip as it could permanently damage the dispenser. DO NOT depress the dispenser while removing the key as it could waste reagent. Discard the shipping key.

STEP 2: Preparing the Dispenser for Use
Remove the Nozzle Cap and place on the Nozzle Cap Holder. Fluid may be present inside the Nozzle Cap. Install the dispenser on the reagent carousel. The Inline Dispenser has been designed to be "Prepared for Use" by the NexES software Version 8.0 or higher. Before each run, the software will detect a new dispenser on the carousel and prime it automatically. Manually priming the dispenser is not necessary and should NEVER be done as it could waste reagent and decrease the number of available dispenses.

Note - All earlier software installations: After removing the shipping key, remove the nozzle cap and CHARGE THE DISPENSER BY RAPIDLY PUMPING 3 to 4 TIMES, keeping the dispenser in an upright position. Charging is only necessary prior to first time use. (See Inspect Prime Before Use section.)

STEP 3: Dispenser Storage & Handling
To insure reliable operation, the dispenser must always be capped when not in use and should NEVER be manually dispensed. (See the Do's and Don't section.)

Do's and Don't-

DO:
1. Check priming chamber and meniscus before each use. (See Inspect Prime Before Use).
2. Store nozzle cap on dispenser. A holder is provided.
3. Cap dispenser when not in use to prevent evaporation. Dispensers mounted on the reagent tray can be capped (from underneath the tray) when not in use.
4. Store dispensers in an upright position in a rack and on the reagent carousel.
5. When mounting the dispenser on the carousel, grasp the coupler to avoid accidental manual dispensing.

DON'T:
1. Do not manually dispense when inverted (upside down). Prime will be lost and may be impossible to restore.
2. Do not manually dispense with the nozzle cap in place. This can permanently damage the dispenser.
3. Do not manually dispense or prime prior to each use. This is not necessary and wastes reagent.
4. Do not hold the barrel in the down position. Fluid can leak from the dispenser when the barrel is depressed.
5. Do not stack carousels with dispensers installed. This can cause the dispensers to leak.

Inspect Prime Before Use:

Remove the nozzle cap and refer to Fig. B.

Dispenser Is Ready For Use When:

1. A meniscus is present in the area shown in Figure B.
2. The priming chamber contains liquid.

If one or both of these conditions is not satisfied, consult Signs of Trouble and What to Do section.

Signs Of Trouble & What To Do:

1. Priming chamber empty. If there is no liquid in the priming chamber, re-prime the dispenser (see Re-Priming the Dispenser section).
2. Meniscus absent. If no meniscus is visible in the nozzle area, manually charge the dispenser once. If this does not resolve the condition, re-prime the dispenser (see Re-Priming the Dispenser section). If condition reoccurs, contact your local Ventana Customer Support Center.
3. Leaking dispenser. External fibers (from clothing or other sources) can cause dispenser to leak. Use in a clean environment.
4. Blocked dispenser. The normal performance characteristics of the dispenser are such that particulates (i.e., fibers, precipitation) could cause a dispenser blockage. A sign of blockage could include higher reagent volume than expected, remaining within the dispenser, after a period of use. Blockage is also evidenced by the failure of the dispenser to yield fluid upon manual dispense, which can be tested by the steps listed in the Re-Priming the Dispenser section. If blockage is suspected (or if foreign material is observed in the dispenser), contact the Ventana Customer Support Center.

NOTE: DO NOT manually dispense or prime the dispenser unless absolutely necessary. Although Ventana pre-filled dispensers have been overfilled to insure a sufficient number of tests, manual dispensing or priming can cause insufficient tests remaining in the dispenser and may cause undesirable staining results.

Consult individual reagent package inserts for information on the utilization of appropriate Quality Control Procedures.

Re-priming The Dispenser:

Once primed, the dispenser should not lose prime if handled correctly. If re-priming is necessary, proceed as follows:
1. Aim the dispenser tip at a waste container. Remove the nozzle cap and depress the barrel (top of the dispenser). This should dispense a drop.
2. If no drop is dispensed, repeat Step 1, above, several times until a drop is ejected.
3. If a drop is ejected, proceed with instructions in Inspect Prime Before Use on this page.
4. If no drop is ejected, or inspection for prime (Step 3) fails, contact your local Ventana Customer Support Center.

Contacting Ventana Technical Consultation Center

If your dispenser does not look or perform as expected, please contact your local Ventana Customer Support Center for advice or return information. Please have the dispenser Lot Number (from the reagent label) handy when you call.

CONTACT INFORMATION:
Ventana Medical Systems, Inc.
1910 Innovation Park Drive
Tucson, Arizona 85755
U.S.A.
+1 520 887-2155
+1 800 227 2155 (USA)

EC REP

Roche Diagnostics GmbH
Sandhofer Strasse 116
D-68305 Mannheim
Germany

Figure A

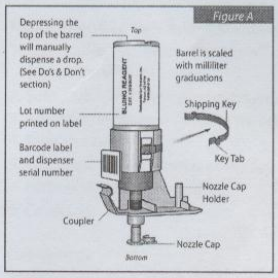
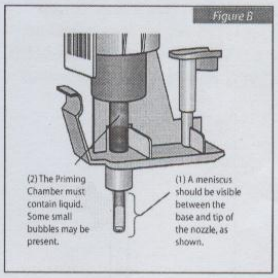
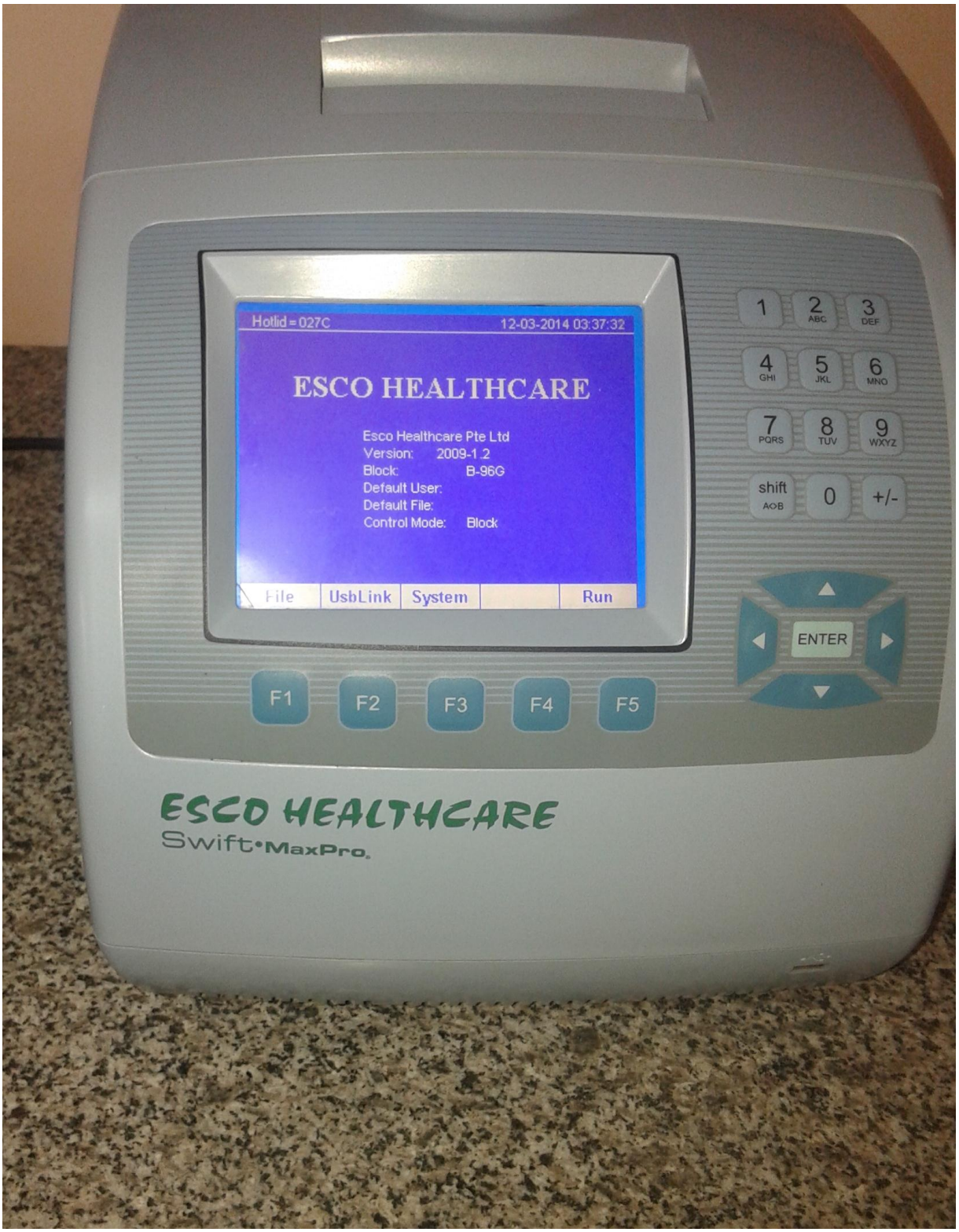


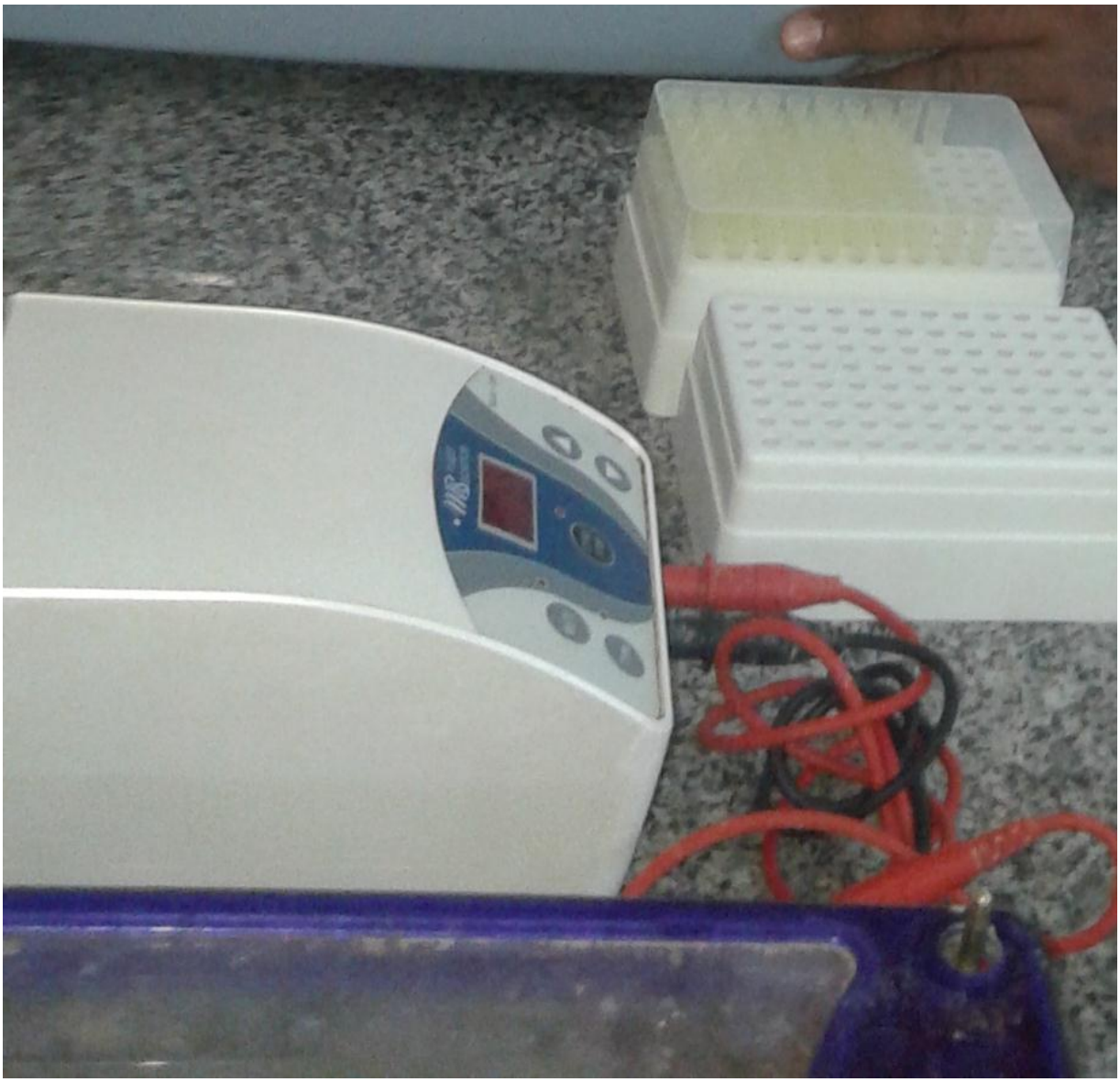
Figure B



Gel documentation



Thermo cycler machine(PCR machine)



Electrophoresis device

Ventana auto stainer machine