TITLE

EBV VCA SPECIFIC IMMUNOGLOBULIN A SERUM ANTIBODY TITRE LEVELS IN NASOPHARYNGEAL CARCINOMA PATIENTS AT THE KENYATTA NATIONAL HOSPITAL.

A CASE CONTROL STUDY

A THESIS SUBMITTED IN PART FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICINE IN EAR, NOSE AND THROAT - HEAD AND NECK SURGERY, AT THE UNIVERSITY OF NAIROBI 2007.

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DECLARATION

This is my original work and has not been presented for a degree in any other university.

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DEDICATION

To my wife Carol and daughter Adela for their immeasurable patience during the course of my studies.

ACKNOWLEDGEMENTS

I would like to thank both my supervisors Professor I. Macharia and Dr E. Njagi for their contribution towards the completion of this study.

ABBREVIATIONS

ENT-HN	Ear, Nose and Throat-Head and Neck
KNH	Kenyatta National Hospital
NPC	Nasopharyngeal Carcinoma
AJCC	American Joint Committee on Cancer
WHO	World Health Organization
EBV	Epstein - Barr virus
VCA	Viral Capsid Antigen
LMP	Latent Membrane Protein
UON	University Of Nairobi

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ABSTRACT

Objective: to investigate and determine the relationship between anti-EBV Immunoglobulin A titre levels and nasopharyngeal carcinoma and its significance in patients diagnosed at Kenyatta National Hospital

Design: case control study

Setting: the study was conducted in the ENT, pathology, and radio-oncology departments of university of Nairobi and KNH.

Methodology: 5 mls of venous blood was collected from 80 patients with a histological diagnosis of NPC, and 80 randomly selected blood donors at the blood transfusion unit. Serum collected was subjected to an ELISA test to determine the titre levels of EBV specific IgA antibodies, levels of EBV titres in the patients with NPC were compared with those of the control group.

Results: show that 35% of patients with NPC had raised titres (titre level> 1:10) while 22.5%, of the control group had raised titres , correlating well with other international studies done elsewhere.

Conclusions: anti-EBV IgA titre levels are significantly raised in patients with nasopharyngeal carcinoma and is not sensitive enough to be used as a diagnostic test.however the titres can be used as an aid in diagnosis in cases where histological diagnosis is unclear, but clinical evidence points in direction of nasopharyngeal carcinoma.

1 INTRODUCTION

The aetiology of nasopharyngeal carcinoma is multi factorial. Implicated factors include EBV which acts as a carcinogen, genetics which determines susceptibility, and environmental factors e.g. eating preserved salted foods.

The titers of immunoglobulin A (IgA) to EBV viral capsule antigen have been used as diagnostic markers in patients suspected to have NPC and to screen for tumour in high risk populations, or relatives of an index patient with a family history of NPC.

This study was to investigate and document local baseline data on the relationship of EBV serology to NPC and to compare them to results of multiple international series which have shown up to 90% of patients with WHO type 2 and 3 NPC having elevated levels of IgA to VCA.

2 BACKGROUND

Epstein-Barr virus (EBV), or human herpesvirus 4, is a gamma-herpes virus that infects more than 95% of the world's population. The most common manifestation of primary infection with this organism is acute infectious mononucleosis, a self-limited clinical syndrome that most frequently affects adolescents and young adults (1). Classic symptoms include sore throat, fever, and lymphadenopathy. Infection with EBV in younger children usually is asymptomatic or mild. However, EBV also is a human tumour virus, which was the first virus to be associated with human malignancy and has been classified as a group 1 carcinogen by the international agency for research on cancer (IARC) (2). Infection with EBV is associated with lymphoproliferative disorders, especially in immunocompromised hosts, and with a variety of tumours including NPC and Burkitts lymphoma.

EBV was discovered in 1964 by electron microscopy of cells cultured from burkitts lymphoma tissue by Epstein, Achong, and Barr (3).Six years later in 1970 EBV DNA was detected in tissues from patients with nasopharyngeal cancer(4).

3 LITERATURE REVIEW

3.1 Aetiology

Aetiology for NPC is still obscure (5, 7, 9). There is a wealth of biologic, biochemical, and immunologic evidence that supports both an association between the Epstein-Barr virus (EBV) and certain forms of nasopharyngeal carcinoma. Genetic, environmental, and viral factors seem to play a part in the genesis of certain histologic types of nasopharyngeal carcinoma (10, 11, 12, and 13). Evidence of EBV-DNA in almost all NPC cells that are studied supports the association of NPC with EBV. Further, the detection of clonal EBV-DNA in NPC suggests that the malignancy is a clonal expansion of a single EBV-infected progenitor cell. This finding indicates that EBV is present

within the cell at the time of malignant transformation and suggests a role for the virus in contributing to the early transformation event. Current knowledge is that the EBV in its latent infection produces LMP1, which aberrantly stimulates cell proliferation.

The contribution of both genetic factors and environmental factors for this disease is reflected in the observation that the incidence of NPC for American-born, second-generation Chinese individuals is lower than that for Chinese-born individuals in China but remains higher than that for white individuals in the United States. Evidence in support of genetic factors is the association of NPC with genotypes HLA-A2 and HLA-Bsin2, which are prevalent in individuals from southern China but rare in Caucasians. Furthermore, abnormalities of multiple chromosomes, including chromosomes 1, 2, 3, 4, 5, 6, 8, 9, 11, 13, 14, 15, 16, 17, 22, and X, have been identified. Possible environmental or cultural factors that may be associated with NPC include the ingestion of Cantonese-style salted fish and preserved foods containing carcinogenic nitrosamines, especially during childhood (7). Molecular basis for the bimodal peaks is still lacking (7).

3.2 Epidemiology/Pathophysiology

Humans are the only known reservoir of EBV. EBV is present in oropharyngeal secretions and most commonly is transmitted through saliva. After initial inoculation, the virus replicates in nasopharyngeal epithelial cells. Cell lysis is associated with release of virions, with viral spread to contiguous structures, including salivary glands and oropharyngeal lymphoid tissues. Further viral replication results in viremia, with subsequent infection of the lymphoreticular system, including the liver, spleen, and B lymphocytes in peripheral blood. Host immune response to the viral infection includes increase in CD8-positive T lymphocytes with suppressor and cytotoxic functions. The T lymphocytes are cytotoxic to the EBV-infected B cells and eventually reduce the number of EBV-infected B lymphocytes to less than 1 per 106 circulating B cells (1).

Primary infection with EBV is followed by latent infection, a characteristic of herpes viruses. After acute EBV infection, latently infected lymphocytes and epithelial cells persist and are immortalized. In vivo, this allows perpetuation of infection, while in vitro,

immortalized cell lines are established. During latent infection, the virus is present in the lymphocytes and oropharyngeal epithelial cells as episomes in the nucleus. These episomes rarely integrate into the cell genome but do replicate with cell division and are passed to subsequent generations of cells. A low rate of viral reactivation occurs within the population of latently infected cells. Epithelial cells are the primary source of new virus in latently infected individuals, infecting B cells as they circulate through the oropharynx.

Two strains labelled EBV-1 and EBV-2 (also known as type A and type B) exist. Although some differences are present in the genes expressed during latent infection, no apparent differences exist in acute illnesses caused by the 2 strains. Both strains are prevalent throughout the world and can simultaneously infect the same person (1).

Knowledge of the structure of EBV and which proteins are expressed during different stages of its life cycle is required to understand the laboratory tests used to determine if an individual has primary acute, convalescent, latent, or reactivation infection. A mature infectious viral particle, which may be present in the cytoplasm of an epithelial cell, consists of a nucleoid, a capsid, and an envelope. The nucleoid contains linear double-stranded viral deoxyribonucleic acid (DNA). It is surrounded by the capsid, an icosahedral constructed of capsomers, which are tubular protein subunits. An envelope derived either from the outer membrane or the nuclear membrane of the host cell encloses the capsid and nucleoid, i.e., the nucleocapsid. The envelope also contains viral proteins that were constructed and placed in the host cell membrane before viral assembly began.

To initiate cellular infection, a viral particle attaches via its major outer envelope glycoprotein, i.e., gp350/220, to the EBV receptor CD21 on a B lymphocyte. The binding site on epithelial cells is not certain but also may be CD21. EBV then is internalized into cytoplasmic vesicles. After fusion of virus envelope with the vesicle membrane, the nucleocapsid is released into the cytoplasm. The nucleocapsid dissolves, the genome is transported to the cell nucleus, and the linear genome then circularizes, forming an episome. The cell then may proceed with either lytic infection with release of infectious virus or latent infection of the host cell. B lymphocytes with latent infection undergo growth transformation (1).

Lytic infection occurs early after primary inoculation. As a result of lytic infection in oral epithelial cells, EBV can be found in the saliva for the first 12-18 months after acquisition. Thereafter, epithelial cells and lymphocytes are latently infected, with a few spontaneously converting, leading to viral replication, host cell lysis and death, and release of mature virions. Thus, virus can be isolated from oral secretions of 20-30% of healthy latently infected individuals at any time. (2)

During latent infection, cell proteins are expressed in 1 of 3 patterns. Type I latency, associated with Burkitt lymphoma, is characterized by expression of only EBV-encoded ribonucleic acids (RNAs), Epstein-Barr early regions (EBERs), and Epstein-Barr nuclear antigen 1 (EBNA1). Type II latency, associated with nasopharyngeal carcinoma, is characterized by expression of 3 latent membrane proteins, LMP1, LMP2A, and LMP2B, plus EBERs and EBNA1. Type III latency is the pattern found in healthy individuals with latent infection. In addition to the EBERs and EBNA1 expressed in type I latency, other nuclear antigens (including EBNA2, EBNA3A, EBNA3B, EBNA3C, and LMP) are expressed in type III latency

The highest incidence of NPC occurs in southern China and Hong (24) Kong. Moderately high incidence is found in Southeast Asian countries, Asian races, North Africans and in Kenyans (5). Low incidence is found in Europeans, Americans and rest of the world. It has been found that when and where the southern Chinese migrate, they retain their high risk for NPC (5). Emigration from high-incidence to low-incidence areas (e.g., United States, Canada) reduces the incidence of nasopharyngeal carcinoma in the first generation, but it is still greater than that in Caucasians. Dickson (6) in a unique, racially balanced series of 209 nasopharyngeal carcinoma cases, found that the incidence of nasopharyngeal carcinoma among Chinese born in China was 118 times the rate in whites and that in Chinese born in North America it was seven times the rate in whites. The biologic behavior of the disease appeared to be the same in Chinese and non-Chinese persons. In Kenya, hospital based studies show that the majority of the patients seen with NPC are from central and eastern province. The male gender is more affected with the male to female ratio of 2.2: 1 (in comparison with global figure of 2.3: 1) (7).

Overall, NPC shows a bimodal age distribution for example in the Chinese 1% occurs before the age of 25 years while majority is seen between 40 and 60 years (8). Mean age in a study done in Saudi Arabia was seen to be 37.5 years while in Ghana it was 24.5 years (7). In Kenya the distribution is bimodal, the first peak between 10 and 20 years and a bigger one between 30 and 40years old (7).

4 CLINICAL PRESENTATION OF NPC

NPC has no characteristic macroscopic features. The lesion may appear ulcerative, infiltrative or be exuberant polypoid tumor. Early asymptomatic and infiltrative carcinoma retains a relatively normal mucosal appearance and the value of screening in such submucosal lesions is unquestionable.

The primary tumour distribution in the nasopharynx is in the following order of decreasing frequency. Lateral wall (especially around the fossa of Rosen Muller and around the eustachian cushions), superior (roof) to posterior wall, more than one wall, anterior wall and floor. More than 80% of tumours are unilateral with the right and left sides being affected equally (5). Complaints of nasopharyngeal carcinoma patients are related to the location of the primary tumor and the degree of spread. Generally, subtle symptoms and signs are confusing to primary care physicians, neurologists, ophthalmologists, and other specialists until the disease has reached advanced stages. Late diagnosis accounts for the poor outcome in many cases. Hearing loss and a lump in the neck are the most common reasons for seeking medical attention (6, 18, 19, 20). The abundant supply of regional lymphatic vessels in the nasopharynx contributes to the high prevalence of cervical metastasis. Approximately 44-57% of patients initially seek medical attention because of a metastatic lymph node that manifests as a neck mass. At the time of diagnosis, 60-85% of patients already have cervical metastasis (5).

Large primary tumors of the nasopharynx obstruct the choana and nasal airway, sometimes causing blood-tinged anterior or postnasal drainage.

Superior extension of tumor through the foramen lacerum, which is an unimpeded pathway near Rosenmüller's fossa into the cranium, leads to cranial nerve involvement (21, 22). In such cases, radiographic evidence of bone destruction at the skull base often exists. Most commonly, cranial nerve (CN) VI is the first to become involved, diplopia

results from external rectus paresis, and the eye signs may be further complicated by involvement of CN III and IV. A characteristic pain high in the neck, facial pain, or facial paresthesia reflects tumor infiltration of CN V. Tumor in the proximity of the jugular foramen leads to paresis or paralysis of CN IX, X, and XI (so-called jugular foramen syndrome). More widespread involvement of the skull base leads to involvement of CN XII. Classically, diagnosis of upward spread of the tumor has been based on (a) the radiographic findings of invasion of the base of the middle fossa, usually in the region of the foramen lacerum, or on (b) involvement of the anterior group of cranial nerves (nerves I to VI, the so-called petro-sphenoid route) or the posterior group of CNs (VII to XII, along the base of the posterior fossa by way of the post-styloid route). Ackerman and Del Regato (23) have described these pathways of spread. Radiographic findings of bone destruction or involvement of either of these two groups of cranial nerves may be considered evidence of superior invasion in most cases (20). All these symptoms are signs of advanced disease most of which are incurable. Unfortunately these features of advanced NPC are characteristic of patients referred to KNH. In order to improve treatment outcome it is imperative that the disease be diagnosed in stage II or I. The earliest symptoms are usually a vague unilateral headache or unilateral facial parasthesia. Most patients however are not impressed by these symptoms and neither seek medical attention nor offer such history to medical attendants. The majority of patients seek medical attention only after the appearance of cervical nodes (24).

Systemic dissemination also occurs more readily in NPC than in other head and neck cancers. The most frequently involved sites are bone, lung, and liver. Distant metastases are present in 5-10% of patients at the initial presentation. Symptoms and signs of nasopharyngeal carcinoma and their frequency at diagnosis in KNH was recorded in a study done by Muchiri (2003) and the results are shown in the following table (24).

Symptom	Frequency	(%)	Symptom	Frequency	(%)
Neck Swelling	100	80.0	Dysphonia	7	5.6
Nasal Blockage	89	71.2	Dysphagia	18	14.4
Epistaxis	56	44.8	Trismus	26	20.8
Nasal Discharge	1	0.8	Proptosis	13	10.4

Nasal Growth	13	10.4	Diplopia	8	6.4
Ear Blockage	25	20	Failing vision	8	6.4
Otalgia/Ear Pain	11	8.8	Facial numbness	14	11.2
Trinitus	17	13.6	Others	24	19.2
Hearing Loss	47	37.6	Total	125	100

5 DIAGNOSTIC EVALUATION

5.1 Examination of the patient

Routine general examination is mandatory. A detailed history should be taken before performing a complete and detailed otolaryngology examination (9, 25).

5.2 Nasopharyngeal examination

The nasopharynx can indirectly be viewed with a mirror. This has many draw backs with the 'functional' channel for examination being about 2 cm only. The procedure is also restricted by pharyngeal reflex, the patient's cooperation and inability to open the mouth. The mirror only gives an "edge view" of the fossa of Rosenmuller.

Biopsy can be taken using mirror examination under general anesthesia with mouth opened by a Boyles-Davies mouth gag and soft palate retracted by nasal-oral catheters. An occult NPC is usually diagnosed by taking blind biopsies from the nasopharynx. Transnasal rigid endoscopy and biopsy under local anesthesia is the preferred method at KNH.

Tissue biopsy for histopathology remains the gold standard for diagnosis of NPC. In transnasal method a blind biopsy is obtained using transnasal Hildyard biopsy forceps or directly using rigid or fibreoptic endoscopy.

The flexible endoscopy gives a good view of the nasal floor, walls of nasopharynx and fossa of Rosenmuller. A biopsy can then be taken using a Hildyard forceps inserted

through the contralateral nostril. This has advantages in that small tumors in any nasopharyngeal quadrant including fossa of Rosenmuller can be biopsied accurately and problems of post biopsy bleeding obscuring the vision is avoided (scope is far away from biopsy site). It can also be used to detect and biopsy post irradiation tumour recurrence beneath a necrotic scab. Contact endoscopic biopsies (after staining 1% methylene blue) are an office procedure that is accurate and allows in vivo diagnosis of NPC (26).

5.3 Value of CT scan in NPC

The nasopharynx can appear deceptively normal when the carcinoma has spread extensively outwards by submucosal infiltration. A CT scan then becomes very helpful in delineating the underlying tumor extension and skull base erosion. CT scanning of the nasophanyx provides a wealth of information that is extremely useful in differentiating malignant from benign disease as well as evaluating the extent and aggressiveness of any inflammatory disease (27).

In a study done to evaluate the value of CT scan in NPC by the department of Radiology UCLA medical center, Los Angeles (1982), all the patients with primary malignant neoplasm of the nasopharynx demonstrated obliteration of one or more parapharyngeal facial planes on CT scanning. A number of squamous cell carcinoma that had spread to the nasophanyx from other sites also demonstrated invasion of the facial planes. Primary lesions of the nasopharynx tend to invade deeply and extend fairly extensively into the deep structures early while secondary disease has a more superficial spread until late in the disease hence CT scan can also be of prognostic value (12). Intravenous contrast given during CT scanning discretely outlines extent of vascular tumours e.g. angiofibromas or glomus jugulare (27).

Contrast enhanced CT scans also help in determining deep node involvement. X-ray findings of bone erosion at the base of skull especially middle cranial fossa usually in the region of the foramen lacerum would give evidence of superior spread of tumour (8). Chest and thoracolumbar X-rays and abdominal ultra sound give information on distant

metastasis. Positron emission tomography has a higher potential of detecting neck node metastasis of NPC than CT scan (28).

5.4 Histopathology

World Health Organization (WHO) Classification for NPC based on light microscopy identifies three distinct histologic types of the primary lesion (14, 15, 16) (See table 1).

Table 1. World Health Organization (WHO) Classification System

WHO Types	Histology Types	Frequency
WHO type 1	Keratinizing squamous cell carcinoma (well-differentiated)	25%
WHO type 2	Nonkeratinizing squamous cell carcinoma (transitional cell carcinomas)	15%
WHO type 3	Undifferentiated carcinoma (lymphoepithelioma and anaplastic carcinomas)	60%

Electron microscopy shows ultrastructural characteristics (desmosomes, tonofibrils) of squamous (epidermoid) carcinomas in the entire spectrum of these tumors, which at one extreme are well-differentiated keratinizing types and at the other extreme are, undifferentiated anaplastic variants. Many studies have shown a preponderance of the anaplastic type (17) (80% in UK). In Kenya, most pathologists do not use the WHO classification and instead use a classification ranging from well differentiated to anaplastic variants. The anaplastic variant could be taken to be synonymous with undifferentiated type. In a recent study anaplastic type was found to comprise 91.6%, squamous cell carcinoma 5.2% and transitional cell carcinoma was 3.2% (7). Another retrospective study done by Oburra at KNH (1988-1992), showed the distribution to be; squamous cell carcinoma 2 (5.9%) and undifferentiated 32 (94%) (n=34) (18).

5.5 Screening

Many studies have shown that NPC is closely associated with EBV. Seroepidemiologic studies have demonstrated that 80-90% of patients with World Health Organization (WHO) type 2 NPC and WHO type 3 NPC have elevated levels of immunoglobulin A (IgA) antibodies to viral capsid antigen (VCA) and early antigen (EA). However, only 10-20% of patients with WHO type 1 NPC have elevated levels of IgA antibodies to VCA (8).

Other serologic tests (which are not as well known) include IgA antibodies directed against, Epstein-Barr (virus) nuclear antigen (EBNA)–1 (found in about 90% of patients with NPC), and immunoglobulin G (IgG) antibodies to the EBV replication activator.

Another useful marker is the EBV gene encoding the latent membrane protein 1 (LMP1). Although the gene that encodes LMP1 (LMP1) is not expressed consistently in all NPC (only about 65%), LMP1 is detected in every NPC cell. Fine-needle aspiration of a neck mass may be useful for the detection of an occult nasopharyngeal primary tumor. The PCR technique can be used to evaluate the aspirate for the presence of EBV-DNA (41). Imaging Studies, which include plain x-rays, CT scans and MRI, are expensive and not suitable for screening purposes

6 TREATMENT

Radiation therapy is the mainstay of treatment, with chemotherapy used in advanced cases. Concurrent cisplatin, 5-fluorouracil, and radiotherapy have been shown to improve survival. Other studies have employed neoadjuvant chemotherapy followed by radiation therapy with improvement in local control or progression-free survival rates. Patients in Kenya are usually diagnosed late; nevertheless they are given full course of radiotherapy with intent of cure.

7 PREVIOUS STUDIES

In a study by Leung et al at the University of Hong Kong in 2003 on application of EBV- DNA and anti-EBV VCA IgA antibodies in nasopharyngeal cancer, 139 NPC patients and 178 healthy individuals were tested for IgA anti-EBV VCA antibodies in their sera. 81% of the NPC patients had positive values while only 4 % of the healthy control subjects had positive values

Neel et al in 1983 did a prospective study on anti- Epstein - Barr virus antibody titres in sera from North American patients with NPC and in control groups. 151 NPC patients and 278 healthy donors had their blood tested for IgA antibodies to EBV.68% of the NPC group had positive titres while only 9% of the healthy donors had elevated titres.

In a study done at mahidol university Bangkok Thailand, ninety one patients with NPC and 164 healthy controls were tested for the presence of IgA anti EBV VCA antibodies. IgA anti-VCA was found in 83.5% of NPC patients and 9.8% of the controls.

8 STUDY JUSTIFICATION

Despite the major advance in optics, tumors of the nasopharynx may still evade the examiner secondary to submucosal spread of tumor, tumor hidden in the fossa of Rosenmuller, or a very small primary. As a result, a normal appearing nasopharynx does not rule out NPC. Biopsy of the nasopharynx may prove challenging for many of the reasons mentioned. Mucosa covering a tumor which has spread submucosally may be histologically normal. Repeated or more aggressive biopsies may be required. Radiologic studies consisting of thin cut CT scans with intravenous contrast are essential in defining the extent of tumor, both as an aid in staging and in determining the radiotherapeutic plan. CT scans may help in detecting very early and hard to locate tumors, although subtle asymmetries in the nasopharynx are common and tumor may be difficult to distinguish from normal variation. Therefore, serologic markers for EBV may play an important role in detection of the disease. Positive serology for EBV in a patient with a cervical metastasis of unknown primary may direct attention toward the nasopharynx.

This study aimed to provide useful evidence of the significance of anti- EBV IgA antibody titre levels in NPC in the Kenyan population.

9 AIM

To find out the relationship of anti EBV VCA IgA antibody titer levels, to NPC and its significance in patients diagnosed at KNH.

10 SPECIFIC OBJECTIVES

1. To determine if anti-EBV antibodies are elevated in NPC patients as compared to healthy subjects.
2. To determine relationship of anti EBV antibody titres to the different histological types of NPC.
3. To determine if anti EBV serological tests can be used as an accurate diagnostic tool for NPC patients in Kenya.

11 STUDY DESIGN

This was a hospital based case control study, at the Kenyatta National hospital, in which 80 NPC patients were the case group while 80 blood donors formed the control group.

11.1 SETTING

Patients

Patients were recruited and their blood samples collected from the ENT-HN and radio oncology departments of KNH while analysis of the samples was done at the department of pathology of the U.O.N immunology laboratory. Patients being recruited from December 2006, to May 2007.

11.2 STUDY POPULATION

All patients attending the above departments who had been histologically diagnosed to have NPC and healthy subjects donating blood at the blood transfusion unit of KNH.

11.3 INCLUSION CRITERIA

A) Study group: Any person with a histological diagnosis of NPC presenting at study sites, irrespective of age that was willing to join the study, and ready to give informed consent.

B) Control group: Healthy blood donors at the KNH blood transfusion unit willing to join the study, irrespective of age and who were willing to give consent to participation in study.

11.4 EXCLUSION CRITERIA

Those patients or blood donors who declined participation in study declining consent as well as any patient who had recurrence of NPC.

11.5 SAMPLE SIZE

The sample size was determined using the statistical formula for case control studies with statistical power (29).

$$n = \frac{(p_o q_o + p_1 q_1)(Z_{1-\alpha/2} + Z_{1-\beta})}{(p_1 - p_o)}$$

Where; p_o = estimated prevalence in cases.

P_1 = estimated prevalence in controls.

$Z_{1-\alpha/2}$ = 5% level of significance (1.96).

$Z_{1-\beta}$ = 80% power (0.84).

The desired n = 79 patients per group.

Therefore total n was 158.

11.6 Study period

The study took a span of six months from December 2006 to May 2007.

12 STUDY METHODS

12.1 Recruitment of study patient

All patients attending the study sites were evaluated for eligibility, and upon satisfying the inclusion criteria were recruited into the study. There was no age limit in the recruiting process. An explanation of the study was given to the patient and consent

obtained. A structured questionnaire was filled by the investigator after a detailed history and physical examination of the patient (see appendix II).

With patient lying in supine position, 5 ml of venous blood was then collected aseptically from the patients forearm, and put in labelled plain sample bottles/test tubes which were put in cold storage, and were run as a batch using l the microwell ELISA serological EBV test kit was performed at the U.O.N immunology department laboratory. IgA anti-EBV VCA titres of $\geq$ 1/10 were considered positive. This cut-off titre has commonly been adopted in previous studies on the marker (see appendix IV).

All the information was recorded in a proforma; the data collected was entered in a computer and analyzed by SPSS statistical package with the help of a statistician.

12.2 LABORATORY METHODS

Washing buffer was prepared by adding distilled water to concentrate. All specimens were brought to room temperature. Assay was then performed and reading of optical density done at 450nm with microwell reader.

The EBV VCA IgA index was calculated by dividing the mean values of each sample by the calibrator mean value (see appendix IV).

12.3 DATA ANALYSIS

All information collected was entered into a patient's assessment form (appendix II). The data was checked for completeness, consistency and accuracy. It was transferred into a coded sheet and analysed using SPSS version 11.0 with the help of a statistician. Statistical analysis was performed using Pearson chi-square or Fisher's exact test (categorical variables) and the *t*-test (continuous variables). $P<0.05$ was regarded as statistically significant. The data was later presented in text, graphs, tables and charts and conclusion and recommendations drawn from the results.

12.4 ETHICAL CONSIDERATION

Study participants were inducted only on voluntary consent by self parent or guardian. Patients who declined to participate were given the same care as those in the study.

There was no extra cost to the participants and the study was approved by the ethical committee of the KNH.

The results of the study will be published and made available for use by members of the medical fraternity

13 STUDY RESULTS

A total of 160 individuals were recruited into the study. 50% of the study population patients were the case study patients with a histological diagnosis of NPC and 50% being normal healthy blood donors. The median age of the study population was 39 years with median age within the case group being 48 years and 31 years for the control group. Majority of candidates in each group were male (75% and 72% respectively).

Majority of NPC patients were in their fifth (34%) and sixth (26%) decade of life while majority of those in the control group were in their third (44%) and fourth (40%) decades of life. Table shows the profile of the study patients.

14 Table1. Sex and age distribution of study population.

	CASES (n= 80)	CONTROLS (N=80)	TOTAL
MALE	60 (75%)	58 (72%)	118 (65%)
FEMALE	20 (25%)	22 (28%)	42 (34.5%)
AGE			
MEAN	45	28	
RANGE	17 to 82	18 to 40	
< 20	3	12	15
21 – 30	6	28	34
31 – 40	12	26	38
41 - 50	27	14	41
51 - 60	21	-	21
> 60	11	-	11
			160

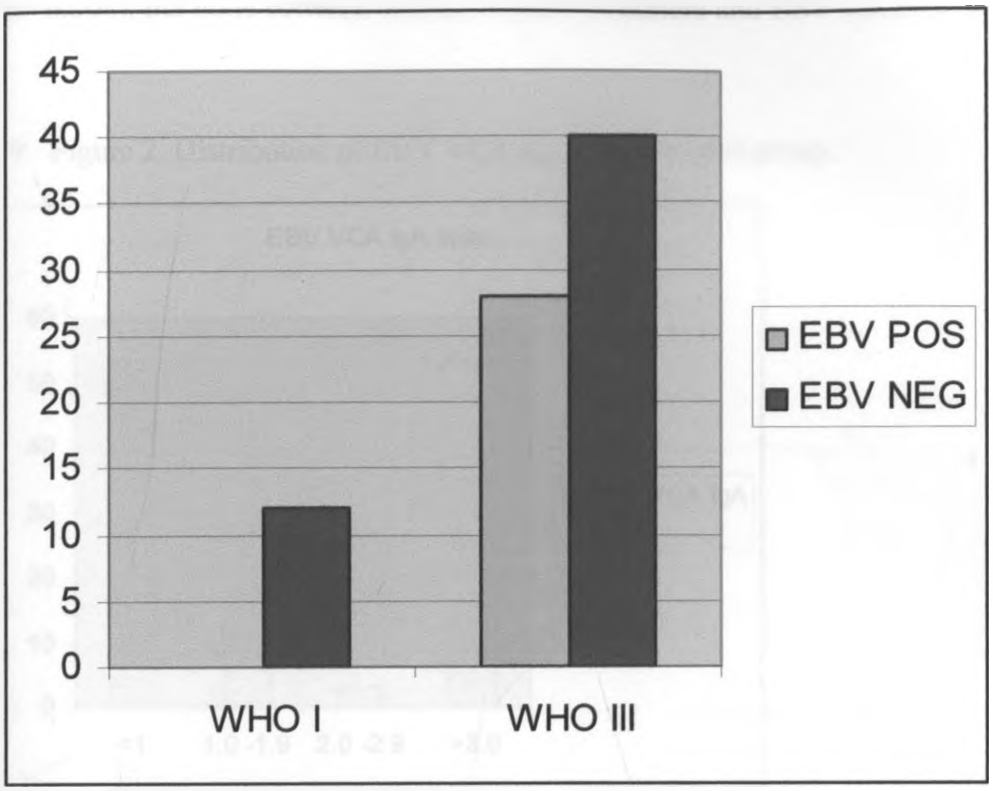
Majority of subjects were males, most subjects in case group were in their fifth decade of life compared to third decade in the control group.

15 Table3. Symptoms and signs in study population.

PRESENTING SYMPTOMS	CASE (%)	CONTROL (%)
nasal	64	-
aural	34	-
oral	16	-
neck	67	-
eyes	10	-
others	13	-
CLINICAL FINDINGS		
nasal	64	-
aural	24	-
oral	11	-
neck	11	-
eyes	6	-
others	5	-

Nasal clinical symptoms and signs predominated followed by aural and oral ones, reflecting the nature of local disease spread.

16 Figure 1. Histological status versus EBV status.



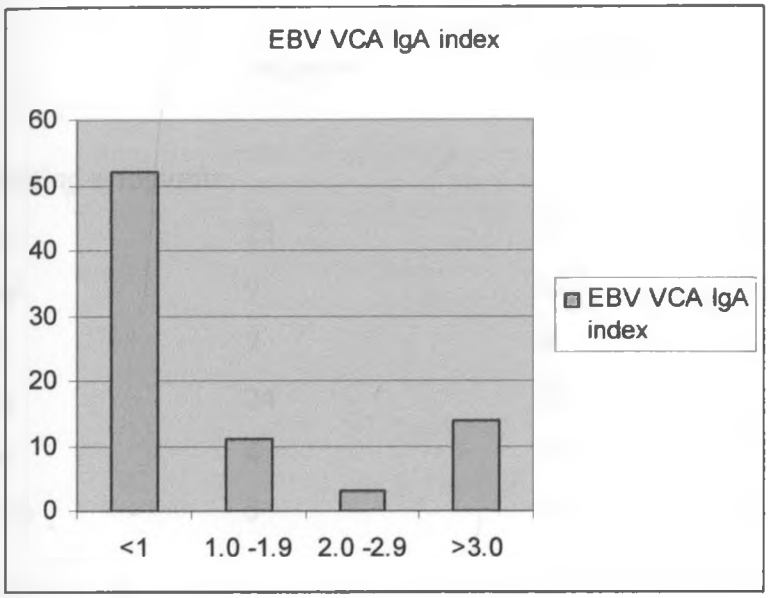
As expected majority of NPC cases were WHO type III. All EBV positive cases were also found in this group.

17 Table 4, Relationship of EBV status with sex and age.

	CASE	CONTROL	P value
MALE	22	15	0.222
FEMALE	6	3	0.177
Age group			
<20	1	2	0.097
21 – 30	2	6	0.083
31 – 40	2	5	0.064
41 – 50	9	3	0.069
51 – 60	8	-	0.075
> 60	6	-	0.087

A strong statistical association was found to exist between WHO type III and EBV status (p= 0.006), but none between chemoradiation treatment and EBV status (p =0.394).

19 Figure 2. Distribution of EBV VCA IgA index in case group.



Majority of patients had EBV VCA IgA index of less than 1.0 (65%).

Table 6 : EBV versus NPC status.

	EBV POSITIVE	EBV NEGATIVE	P VALUE
NPC POSITIVE	28	52	0.02
NPC NEGATIVE	18	62	

35% of the case group and 18% of the control group had elevated EBV VCA IgA levels. There was a statistically significant relationship between elevated EBV VCA IgA levels and presence of NPC (P= 0.02).

No statistical significant association was found between age and EBV status.

18 Table 5, Relationship of the EBV with NPC clinical presentations.

	Negative	Positive	P value
Presenting symptoms			
Nasal	23	41	0.725
Aural	9	25	0.169
Oral	7	9	0.412
Neck	24	43	0.727
Eyes	4	6	0.723
Others	5	8	0.775
Clinical findings			
Nasal	26	38	0.695
Aural	6	18	0.238
Oral	5	6	0.400
Neck	15	52	0.691
Eyes	4	7	0.743
Others	2	4	0.512
Histological status			
WHO I	0	12	-
WHO II	0	0	-
WHO III	28	40	0.006
Chemoradiation	2	7	0.394

No significant association was found between clinical presentation of disease and EBV status.

20 DISCUSSION

There was no statistically significant relationship between EBV status and sex of the subject (p=0.222) age of the subject, status of chemoradiation treatment (p=0.394) or stage of Nasopharyngeal carcinoma (p =2.9).

The results of this study show that EBV VCA IgA titre levels were elevated in NPC patients compared to healthy individuals. Although the percentages in the case group were comparatively lower in comparison to previous studies done in other parts of the world, and relatively higher in the healthy control group, there is still a statistically significant relationship between the two values (p= 0.02)

Previous studies done in South East Asia and North America have shown prevalence rates of elevated EBV VCA IgA levels of between 68 and 83 percent in NPC patients, and between 4 and 10% in healthy control groups.

EBV VCA IgA levels have been found to decline in patients with NPC receiving chemo radiation and are used in other parts of the world as a follow up measure to gauge response to treatment as well as to detect persistence or recurrence of tumour. In this study 29% of patients receiving chemoradiation as compared to 37% of those who had not received chemoradiation had elevated titres. While this reflects the expected trend, the numbers of patients receiving chemoradiation included in the study were very small, only 11.25% of the case group.

It is also important to note that details of the stage of chemoradiation when patient was assessed was not recorded and therefore no correlation can be accurately drawn as to the effect of chemoradiation to EBV status in this study.

An argument could be made as to whether it would have been better to age match the study groups, but the fact that most primary infections happen in childhood tends to standardize the issue of exposure. More over it would necessitate choosing the controls differently since most blood donors are usually in their second and third decades of life,

and rarely in their fourth and fifth decades where NPC was found to be most prevalent in this study.

Positive EBV status in the control group was found to be mostly in the third (40%) and fourth (33%) decade of life, while in the case group it was mainly in the fifth (32%) and sixth (28%) decades in which NPC incidence also predominated. There was however no statistical significance relationship, between age and EBV status in either group with P values of 2.90 and 3.44 respectively.

All patients with elevated levels of EBV VCA IgA in the nasopharyngeal cancer positive group had the WHO type III histological type, showing a significant relationship between titre levels and histological type, although this histological type was also the most predominant.
WHO type one has not been shown to be associated with elevated anti-EBV titres in previous studies, a fact reflected in this study, although the numbers involved were quite small only 12 patients(15%).

Previous studies have shown a bi-modal peak in incidence of NPC.In this study the peak incidence was mainly in the fourth and fifth decades.

21 CONCLUSION

EBV VCA IgA titre levels are elevated in patients with nasopharyngeal carcinoma presenting at Kenyatta National hospital

Elevated EBV VCA IgA levels were found predominantly in patients with nasopharyngeal carcinoma of the anaplastic type (WHO type III), which is also the commonest histological variety in the local Kenyan population

While there is a significant statistical difference in titre levels between patients with NPC and those without, EBV VCA IgA titre measurement can only be used as an adjunctive investigative measure to confirm diagnosis, while tissue histology remains the gold standard.

22 RECOMMENDATIONS

1. Serological tests for EBV VCA IgA titre levels should be used more often in as an aid to reaching a diagnosis in suspected NPC cases where repeated tissue histology is negative, this would reduce delay in diagnosis as well as reduce cost to the patient.
2. Further research needs to be done in our local setting to determine if serological tests can be used to as a follow up measure in patients already treated with chemo- radiation, to detect persistent or recurrent tumours.
3. A study with a larger sample size needs to be done to corroborate the findings of the present one in view of the relatively low EBV VCA IgA titre levels in NPC patients in comparison with previous studies in other parts of the world

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24 APPENDIX 1- GENERAL PATIENT INFORMATION

This study includes only patients who agree to take part. Please take your time to make your decision. We aim to find out the relationship of infection with the EBV virus to cancer of the nasopharynx.the results of this study may help us to detect early stages of the cancer and also help assessing effectiveness of treatment.

WHAT IS INVOLVED IN THE STUDY?

If you are recruited in to the study a history about your illness will be taken and physical examination of your head and neck will be done. The results will be recorded on a form. A sample of blood approximately 2 mls will be removed from your hand.The EBV serology test will be carried out on this sample and results recorded

WHAT ARE THE RISKS OF THE STUDY?

Although some discomfort may be experienced while introducing the needle into your hand, there are no added risks peculiar to the procedure involved.

WILL I BE PENALISED FOR NOT PARTICIPATING IN THE STUDY?

There will be no penalties at all. Those who chose not to participate in the study will be given the same treatment and attention as those who chose to participate.

ARE THERE BENEFITS IN TAKING PART IN THE STUDY? If you agree to take part in this study, there may or may not be direct medical benefits to you. We hope the information learned from this study will benefit other patients with NPC. You may choose not to participate in this study and you will still get the treatment just like a person who is in the study.

WHAT ABOUT CONFIDENTIALITY?

Efforts will be made to keep your personal information confidential. Records of your history, physical examination and blood serology results while in the study will be kept in a confidential form.

WHAT IS THE COST? Taking part in this study will not lead to any added cost on top of what you pay to the hospital for your usual treatment. If you are among the control group no extra cost will be incurred.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

Taking part in this study is voluntary. You may choose not to take part or may leave the study any time. Leaving the study will not result in any penalty or loss of benefits that you are entitled to.

WHAT DO WE DO WITH THE INFORMATION WE GET?

1) The information we get may not be of very immediate benefit to you but may help us to diagnose and treat patients with this cancer early.

2) Like all scientific information we will seek to share our findings with other people undertaking similar studies. Therefore we may publish our findings in scientific journals or present them at meetings.

3) If you require discussing this matter with your family, friend or associates you are free to do so and we will be ready to answer any question. If you are satisfied with our explanation and are willing to participate then please fill and sign the consent below.

CONSENT FOR STUDY

I..... ID No.....

Study No.....

Of.....do hereby consent to be included in the study on the relationship of Epstein barr virus to nasopharyngeal cancer at KNH, and to have my blood withdrawn.

The nature of the study has been explained to me by Dr.....

And I have not been promised any material gain to be included in this study

Signed.....(self)

CONSENT FOR UNDER 18 YEAR OLDS

I ..... ID No.....

Study No.....

Parent/guardian of.....

Do hereby give consent for my child to be included in the study on the relationship of EBV virus to nasopharyngeal cancer at KNH,and to have his/her blood withdrawn.

The nature of the study has been explained to me by Dr.....

I have not been promised any material gain to be included in this study

Signed.....(parent/guardian).

MAELEZO KWA WAHUSIKA WA UTAFITI

Utafiti huu ni juu ya uhusiano kati ya virusi vya EBV na saratani ya koo. Wanaokubali tuu ndio watakao husishwa, wala hakuna kulazimishwa.Utafiti huu unaweza kuongeza ujuzi wetu juu ya saratani ya koo na njia za kuugundua mapema.

TARATIBU YA UTAFITI

Wahusilka watatolewa damu kiasi kidogo kutoka kwenye mkono kwa kudungwa sindano.Pia Daktari atawauliza maswalijuu ya hali yao ya afya ya hapo mbeleni.

KUNA HATARI GANI

Mhusika atahisi uchungu kidogo anapodungwa sindano, zaidi ya hio hakuna hatari au madhara nyingine.

JE NIKIKATAA KUHUSIKA?

Hakuna adhbu yeyte kwa kukataa.Matibabu sawia yatapewa kwa wote wahusika na wasiohusika.

KUNAFAIDA GANI KWANGU

Matokeo ya utafiti yanaweza kukufaidi kiafya au la.Tunatarajia kutumia matokeo yetu kunufaisha wagonjwa wote wa saratani ya koo sasa na baadaye.

NITALIPA PESA ZA ZIADA?

La! Malipo ni yale ya kawaida ya hospitali kwa wote. Wale walio kwenye kundi lisilohitaji matibabu hawata lipa chochote.

MATOKEO YA UTAFITI YATAENDA WAPI?

Matokeo yanaweza kunukuliwa kwenye gazeti za kisayansi ilikunufaisha uchunguzi ju ya saratani duniani,lakini habari za mhusika binafsi yatawekwa siri.

Kama umeridhika na maelezo yetu na unakubali kuhusika, jaza fomu ya kukubali kisha upige sahihi hapo chini.

KIBALI CHA UTAFITI

Mimi..... ID No.....

Numbari ya utafiti.....

Anwani.....nimekubali kuhusishwa katika utafiti huu juu ya uhusiano wa virusi vya EBV na saratani ya koo katika
hospitali kuu ya Kenyatta.Nimepewa maelezo kamili kuhusu utafiti huu na
Dr .....Hakuna malipo yoyote nitkayo pewa kwa kuhusika katika utafiti huu

Sahihi.....(Mwenyewe)

KIBALI CHA UTAFITI KWA WATOTO

Mimi..... mzazi/msimamizi wa.....

ID No.....

Numbari ya utafiti.....

Anwani..... nimemkubalia mtoto wangu ahusishwe katika utafiti huu juu ya uhusiano wa virusi vya EBV na saratani ya koo,katika hospitali kuu ya Kenyatta.Nimepewa maelezo kamili kuhusu utafiti huu na Dr.....

Hakuna malipo yoyote nitakayo pewa kwakuhusika katika utafiti huu.

Sahihi.....(mzazi/msimamizi).

25 APPENDIX II - PROFORMA FOR THE STUDY

Patient's Name :.....

Study No:.....

Sex: ..... Age (in years).....

IP.NO. ..... Date.....

Staging of tumor----------

SYMPTOMS: DURATION

NASAL yes no

Nasal blockage ☐ ☐ ----------

Epistaxis ☐ ☐ ----------

Growth ☐ ☐ ----------

FB sensation ☐ ☐ ----------

Others ----------

AURAL

Feeling of blockage ☐ ☐ ----------

Hearing loss ☐ ☐ ----------

Tinnitus ☐ ☐-- ----------

Ear discharge ☐ ☐ ----------

Growth ☐ ☐- ----------

Others----------

ORAL

Growth (mass) ☐ ☐ ----------

Trismus ☐ ☐ ----------

Difficulty in swallowing -- ☐ ☐ ----------

Pain on swallowing ☐ ☐- ----------

Others ----------

NECK

Growth/Swelling ☐ ☐ ----------

Difficulty in breathing ☐ ☐ ----------

Others----------

EYES

Protrusion ☐ ☐ ----------

Pain ☐ ☐ ----------

Double vision ☐ ☐ ----------

Other symptoms: ----------

SIGNS AT PRESENTATION.

ENT-HN EXAMINATION.

NECK

Nodes Present ☐

Absent ☐

If present, state site i.e. Neck level I, II, III, IVor V ----------

Size: Less than 6cm ☐

More than 6 cm ☐

Multiplicity: single ☐

Multiple ☐

NOSE:

Nasal endoscopy. Rigid ☐

Flexible ☐

NASOPHARYNX:

Lateral walls

Right----------

Left ----------

Roof ----------

Posterior wall ------

Other signs----------

EARS:

Auditory meatus ----------

Tympanic membrane ----------

Tuning fork tests;

Rinnes: ----------Weber: ----------

THROAT: Oral mucosa ----------

Tongue ----------

Oropharynx ----------

Soft palate ----------

Cranial nerve palsy ----------

Respiratory system ----------

+Ve -Ve

Histology results ☐ ☐

If positive, state the type;

Received treatment; yes ----------

No ----------

Type of treatment recommended; ----------

26 APPENDIX III - STAGING (AJCC, 1997)

PRIMARY TUMOR (T)

TX Primary tumour cannot be assessed

T0 No evidence of primary tumour

Tis Carcinoma in situ

Nasopharynx sites

Postero-superior walls

Lateral walls

Inferior (anterior) wall.

Staging primary tumor (T)

T1 Tumor confined to the nasopharynx

T2 Tumor extends to soft tissues of oropharynx and or nasal fossa

T2a without parapharyngeal extension

T2b with parapharyngeal extension

T3 Tumor invades bony structures and/or paranasal sinuses

T4 Tumor with intracranial extension and/or involvement of cranial nerves, infratemporal fossa, hypopharynx, or orbit.

REGIONAL LYMPH NODES (N)

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Unilateral metastasis in lymph node(s), 6 cm or less in greatest dimension, above the supraclavicular fossa

N2 Bilateral metastasis in lymph node(s), 6 cm or less in greatest dimension, above the supraclavicular fossa

N3 Metastasis in a lymph node(s)

N3a greater than 6 cm in dimension

N3b in the supraclavicular fossa

DISTANT METASTASIS (M)

MX Distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis

27 APPENDIX IV - SPECIMEN COLLECTION AND HANDLING

1. Collect blood specimens and separate the serum.

2. Specimens may be refrigerated at 2 - 8o C for up to seven days or frozen for up to six months. Avoid repetitive freezing and thawing of serum sample.

PREPARATION FOR ASSAY

1. Prepare 1x washing buffer.

Prepare washing buffer by adding distilled or deionized water to 10x wash concentrate to make a final volume of 1 liter.

2. Bring all specimens and kit reagents to room temperature (20 - 25o C) and gently mix.

ASSAY PROCEDURE

1. Place the desired number of coated strips into the holder.

2. Prepare 1:20 dilutions by adding 10 ml of the samples, negative control, positive control, and calibrator to 200 ml of absorbent solution. Mix well.

3. Dispense 100 ml of diluted sera, calibrator, and controls into the appropriate wells. For the reagent blank, dispense 100 ml of absorbent solution in 1A well position. Tap the holder to remove air bubbles from the liquid and mix well. Incubate for 30 minutes at room temperature.

4. Remove liquid from all wells. Repeat washing three times with washing buffer.

5. Dispense 100 ml of enzyme conjugate to each well and incubate for 30 minutes at room temperature.

6. Remove enzyme conjugate from all wells. Repeat washing three times with washing buffer.

7. Dispense 100 ml of TMB Chromogenic Substrate to each well and incubate for 30 minutes at room temperature.

8. Add 100 ml of 2 N HCl to stop reaction.

Make sure there are no air bubbles in each well before reading.

9. Read O.D.(optical density) at 450 nm with a microwell reader.

CALCULATION OF RESULTS

1. Calculate the mean of duplicate calibrator value xc.

2. Calculate the mean of duplicate positive control, negative control, and patient samples.

3. Calculate the EBV-VCA IgA Index of each determination by dividing the mean values of each sample by calibrator mean value, xc.

Example of typical results:

Calibrator O.D. = 0.718, 0.704 xc = 0.711

Cut-off calibrator EBV-VCA IgA Index = 1.0

Patient sample O.D. = 0.991, 0.956 xs = 0.974

EBV-VCA IgA Index = 0.974 / 0.711 = 1.37

QUALITY CONTROL

The test run may be considered valid provided the following criteria are met:

1. The O.D. value of the reagent blank against air from a microwell reader should be less than 0.150.

2. If the O.D. value of the Calibrator is lower than 0.250, the test is not valid and must be repeated.

3. The EBV-VCA IgA Index for Negative and Positive Control should be in the range stated on the labels.

INTERPRETATION

Negative: EBV-VCA IgA Index of 0.90 or less is seronegative for IgA antibody to EBV-VCA virus.

Equivocal: EBV-VCA IgA Index of 0.91 - 0.99 are equivocal. Sample should be retested.

Positive: EBV-VCA IgA Index of 1.00 or greater.

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Ref: KNH-ERC/ 01/ 3990

15th December 2006

Dr. Henry Nyawanda
Dept. of Surgery
School of Medicine
University of Nairobi

Dear Dr. Nyawanda

RESEARCH PROPOSAL: "EBV VCA SPECIFIC IgA SERUM ANTIBODY TITRE LEVELS IN NASOPHARYNGEAL CARCINOMA PATIENTS AT THE K.N.H" (P191/092006)

This is to inform you that the Kenyatta National Hospital Ethics and Research Committee has reviewed and **approved** your revised research proposal for the period 15th December 2006 – 14th December 2007.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimen must also be obtained from KNH-ERC for each batch.

On behalf of the Committee, I wish you fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of database that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

PROF A N GUANTAI
SECRETARY, KNH-ERC

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The Deputy Director CS, KNH
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